

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

# 12

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

5808

(51) International Patent Classification 6 : <b>C12N 9/16 // (C12N 9/16, C12R 1:645)</b>		A1	(11) International Publication Number: <b>WO 98/28409</b> (43) International Publication Date: <b>2 July 1998 (02.07.98)</b>
(21) International Application Number: <b>PCT/DK97/00568</b>		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: <b>15 December 1997 (15.12.97)</b>			
(30) Priority Data:			
1480/96	20 December 1996 (20.12.96)	DK	
1481/96	20 December 1996 (20.12.96)	DK	
0301/97	18 March 1997 (18.03.97)	DK	
0529/97	7 May 1997 (07.05.97)	DK	
1388/97	1 December 1997 (01.12.97)	DK	
(71) Applicant: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).		Published <i>With international search report.</i>	
(72) Inventors: LASSEN, Søren, Flensted; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). BECH, Lisbeth; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). OHMANN, Anders; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). BREINHOLT, Jens; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). FUGLSANG, Claus, Crone; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK).			

(54) Title: PHYTASE POLYPEPTIDES

(57) Abstract

The present invention relates to isolated polypeptides having phytase activity, the corresponding cloned DNA sequences, a process for preparing such polypeptides, and the use thereof for a number of industrial applications. In particular, the invention relates to phytases derived from the phylum Basidiomycota, phytases of certain consensus sequences and fungal 6-phytases.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**TITLE: Phytase polypeptides****FIELD OF INVENTION**

The present invention relates to isolated polypeptides having 5 phytase activity, the corresponding cloned DNA sequences, a method of producing such polypeptides, and the use thereof for a number of industrial applications. In particular, the invention relates to phytases derived from the phylum Basidiomycota, phytases of certain consensus sequences and 10 fungal 6-phytases.

**BACKGROUND OF THE INVENTION**

Phytic acid or myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (or for short myo-inositol hexakisphosphate) is the 15 primary source of inositol and the primary storage form of phosphate in plant seeds. In fact, it is naturally formed during the maturation of seeds and cereal grains. In the seeds of legumes it accounts for about 70% of the phosphate content and is structurally integrated with the protein bodies as 20 phytin, a mixed potassium, magnesium and calcium salt of inositol. Seeds, cereal grains and legumes are important components of food and feed preparations, in particular of animal feed preparations. But also in human food cereals and legumes are becoming increasingly important.

25

The phosphate moieties of phytic acid chelates divalent and trivalent cations such as metal ions, i.a. the nutritionally essential ions of calcium, iron, zinc and magnesium as well as the trace minerals manganese, copper and molybdenum.

30

Besides, the phytic acid also to a certain extent binds proteins by electrostatic interaction. At a pH below the isoelectric point, pI, of the protein, the positively charged protein binds directly with phytate. At a pH above pI, the 35 negatively charged protein binds via metal ions to phytate.

Phytic acid and its salts, phytates, are often not metabolized, since they are not absorbable from the gastro intestinal system, i.e. neither the phosphorous thereof, nor the chelated

metal ions, nor the bound proteins are nutritionally available.

Accordingly, since phosphorus is an essential element for the growth of all organisms, food and feed preparations need to be 5 supplemented with inorganic phosphate. Quite often also the nutritionally essential ions such as iron and calcium, must be supplemented. And, besides, the nutritional value of a given diet decreases, because of the binding of proteins by phytic acid. Accordingly, phytic acid is often termed an 10 anti-nutritional factor.

Still further, since phytic acid is not metabolized, the phytate phosphorus passes through the gastrointestinal tract of such animals and is excreted with the manure, resulting in an 15 undesirable phosphate pollution of the environment resulting e.g. in eutrophication of the water environment and extensive growth of algae.

Phytic acid or phytates, said terms being, unless otherwise 20 indicated, in the present context used synonymously or at random, are degradable by phytases.

In most of those plant seeds which contain phytic acid, endogenous phytase enzymes are also found. These enzymes are 25 formed during the germination of the seed and serve the purpose of liberating phosphate and, as the final product, free myo-inositol for use during the plant growth.

When ingested, the food or feed component phytates are in 30 theory hydrolyzable by the endogenous plant phytases of the seed in question, by phytases stemming from the microbial flora in the gut and by intestinal mucosal phytases. In practice, however the hydrolyzing capability of the endogenous plant phytases and the intestinal mucosal phytases, if existing, is 35 far from sufficient for increasing significantly the bioavailability of the bound or constituent components of phytates. However, when the process of preparing the food or feed involve germination, fermentation or soaking, the endogenous phytase might contribute to a greater extent to the

degradation of phytate.

In ruminant or polygastric animals such as horses and cows the gastro intestinal system hosts microorganisms capable of 5 degrading phytic acid. However, this is not so in monogastric animals such as human beings, poultry and swine. Therefore, the problems indicated above are primarily of importance as regards such monogastric animals.

10 The production of phytases by plants as well as by microorganisms has been reported. Amongst the microorganisms, phytase producing bacteria as well as phytase producing fungi are known.

15 From the plant kingdom, e.g. a wheat-bran phytase is known (Thomlinson et al, Biochemistry, 1 (1962), 166-171). An alkaline phytase from lilly pollen has been described by Barrientos et al, Plant. Physiol., 106 (1994), 1489-1495.

20 Amongst the bacteria, phytases have been described which are derived from *Bacillus subtilis* (Paver and Jagannathan, 1982, Journal of Bacteriology 151:1102-1108) and *Pseudomonas* (Cosgrove, 1970, Australian Journal of Biological Sciences 23:1207-1220). Still further, a phytase from *E. coli* has been 25 purified and characterized by Greiner et al, Arch. Biochem. Biophys., 303, 107-113, 1993). However, this enzyme is probably an acid phosphatase.

Phytase producing yeasts are also described, such as  
30 *Saccharomyces cerevisiae* (Nayini et al, 1984, Lebensmittel Wissenschaft und Technologie 17:24-26. However, this enzyme is probably a myo-inositol monophosphatase (Wodzinski et al, Adv. Appl. Microbiol., 42, 263-303). AU-A-24840/95 describes the cloning and expression of a phytase of the yeast *Schwanniomyces* 35 *occidentalis*.

There are several descriptions of phytase producing filamentous fungi, however only belonging to the fungal phylum of Ascomycota (ascomycetes). In particular, there are several

references to phytase producing ascomycetes of the *Aspergillus* genus such as *Aspergillus terreus* (Yamada et al., 1986, *Agric. Biol. Chem.* 322:1275-1282). Also, the cloning and expression of the phytase gene from *Aspergillus niger* var. *awamori* has been described (Piddington et al., 1993, *Gene* 133:55-62). EP 0 420 358 describes the cloning and expression of a phytase of *Aspergillus ficuum* (*niger*). EP 0 684 313 describes the cloning and expression of phytases of the ascomycetes *Myceliophthora thermophila* and *Aspergillus terreus*.

10

#### NOMENCLATURE AND POSITION SPECIFICITY OF PHYTASES

In the present context a phytase is an enzyme which catalyzes the hydrolysis of phytate (*myo*-inositol hexakisphosphate) to (1) *myo*-inositol and/or (2) mono-, di-, tri-, tetra- and/or 15 penta-phosphates thereof and (3) inorganic phosphate. In the following, for short, the above compounds are sometimes referred to as IP<sub>6</sub>, I, IP<sub>1</sub>, IP<sub>2</sub>, IP<sub>3</sub>, IP<sub>4</sub>, IP<sub>5</sub> and P, respectively. This means that by action of a phytase, IP<sub>6</sub> is degraded into P + one or more of the components IP<sub>5</sub>, IP<sub>4</sub>, IP<sub>3</sub>, 20 IP<sub>2</sub>, IP<sub>1</sub> and I. Alternatively, *myo*-inositol carrying in total n phosphate groups attached to positions p, q, r,... is denoted Ins(p,q,r,...)P<sub>n</sub>. For convenience Ins(1,2,3,4,5,6)P<sub>6</sub> (phytic acid) is abbreviated PA.

25 According to the Enzyme nomenclature database ExPASy (a repository of information relative to the nomenclature of enzymes primarily based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) describing each type 30 of characterized enzyme for which an EC (Enzyme Commission) number has been provided), two different types of phytases are known: A so-called 3-phytase (*myo*-inositol hexaphosphate 3-phosphohydrolase, EC 3.1.3.8) and a so-called 6-phytase (*myo*-inositol hexaphosphate 6-phosphohydrolase, EC 3.1.3.26). The 3- 35 phytase hydrolyses first the ester bond at the 3-position, whereas the 6-phytase hydrolyzes first the ester bond at the 6-position.

Inositolphosphate nomenclature

Considering the primary hydrolysis products of a phytase acting on phytic acid, some of the resulting esters are diastereomers and some are enantiomers. Generally, it is easier to discriminate between diastereomers, since they have different physical properties, whereas it is much more difficult to discriminate between enantiomers which are mirror images of each other.

Thus,  $\text{Ins}(1,2,4,5,6)\text{P}_5$  (3-phosphate removed) and  $\text{Ins}(1,2,3,4,5)\text{P}_5$  (6-phosphate removed) are diastereomers and easy to discriminate, whereas  $\text{Ins}(1,2,4,5,6)\text{P}_5$  (3-phosphate removed) and  $\text{Ins}(2,3,4,5,6)\text{P}_5$  (1-phosphate removed) are enantiomers. The same holds true for the pair  $\text{Ins}(1,2,3,4,5)\text{P}_5$  (6-phosphate removed) and  $\text{Ins}(1,2,3,5,6)\text{P}_5$  (4-phosphate removed). Accordingly, of the 6 penta-phosphate esters resulting from the first step of the phytase catalyzed hydrolysis of phytic acid, you can only discriminate easily between those esters in which the 2-, 3-, 5- and 6-phosphate has been removed, i.e. you have four diastereomers only, each of the remaining two esters being an enantiomer of one each of these compounds (4- and 6- are enantiomers, as are 1- and 3-).

Use of lowest-locant rule

It should be noted here, that when using the notations  $\text{Ins}(2,3,4,5,6)\text{P}_5$  and  $\text{Ins}(1,2,3,5,6)\text{P}_5$ , a relaxation of the previous recommendations on the numbering of the atoms of myo-inositol has been applied. This relaxation of the lowest-locant rule is recommended by the Nomenclature Committee of the International Union of Biochemistry (Biochem. J. (1989) 258, 1-2) whenever authors wish to bring out structural relationships.

In this lowest-locant rule, the L- and D-nomenclature is recommended: Inositolphosphate, phosphate esters of myo-inositol, are generally designated 1D- (or 1L-) - $\text{Ins}(r,s,t,u,w,x)\text{P}_n$ , n indicating the number of phosphate groups and the locants r,s,t,u,w and x, their positions. The positions are numbered according to the Nomenclature Committee

of the International Union of Biochemistry (NC-IUB) cited above (and the references herein), and 1D or 1L is used so as to make a substituent have the lowest possible locant or number ("lowest-locant rule"). Accordingly, 1L-myo-inositol-1-phosphate (1L-Ins(1)P, an intermediary product in the biosynthesis of inositol) and 1D-myo-inositol-1-phosphate (1D-Ins(1)P, a component of phospholipids), are numbered as it is apparent from Fig. 38.

10 Phytase specificity

As said above, phytases are divided according to their specificity in the initial hydrolysis step, viz. according to which phosphate-ester group is hydrolyzed first.

15 As regards the specificity of known phytases, plant phytases are generally said to be 6-phytases. However the lilly pollen phytase is said to be a 5-phytase. The microorganism derived phytases are mainly said to be 3-phytases. E.g. the ExPASy database mentioned above refers for 3-phytases to four  
20 phytases of *Aspergillus awamori* (strain ALK0243) and *Aspergillus niger* (strain NRRL 3135) (Gene 133:55-62 (1993) and Gene 127:87-94 (1993)).

Using now the D-/L-notation (in which the D- and L-  
25 configuration refer to the 1-position), the wheat-bran phytase hydrolyzes first the phosphate ester group in the L-6 position, whereas the 3-phytases hydrolyzes first the phosphate ester group in position D-3.

30 The specificity can be examined in several ways, e.g by HPLC or by NMR spectroscopy. These methods, however, do not immediately allow the discrimination between hydrolysis of e.g. the phosphate-ester groups in positions D-6 and L-6, since the products of the hydrolysis, D-Ins(1,2,3,4,5)P<sub>5</sub> and L-  
35 Ins(1,2,3,4,5)P<sub>5</sub>, are enantiomers (mirror images), and therefore have identical NMR spectres.

In other words, in the present context a 6-phytase means either of a L-6- or a D-6-phytase or both, viz. a phytase

being a L-6-phytase, a D-6-phytase or a ((D-6-)+(L-6-))-phytase (having both activities). The latter is sometimes also designated D/L-6-phytase.

##### 5 SUMMARY OF THE INVENTION

It is an object of the present invention to provide alternative phytases, in particular with superior properties such as increased heat stability or faster release of phosphate from phytate, and which can be produced in commercially useful 10 quantities.

The present inventors have surprisingly found a whole sub-family of fungal phytases of interesting properties. This sub-family of phytases is characterized by having a high degree of 15 conserved regions or partial sequences in common (consensus sequences). Representatives of this sub-family have turned up to be advantageous as compared to known phytases as regards various enzyme properties, such as e.g. position specificity and specific activity.

20

It is presently contemplated that the phytase consensus sequences of the present invention are common to all basidiomycete phytases.

25 In the present context a basidiomycete means a microorganism of the phylum Basidiomycota. This phylum of Basidiomycota is comprised in the fungal kingdom together with e.g. the phylum Ascomycota ("ascomycetes"). Reference can be had to Jülich, 1981, Higher Taxa of Basidiomycetes; Ainsworth & Bisby, 1995, 30 Dictionary of the Fungi; Hansen & Knudsen (Eds.), Nordic Macromycetes, vol. 2 (1992) and 3 (1997). Alternatively, a fungal taxonomy data base (NIH Data Base (Entrez)) is available via the internet on World Wide Web at the following address: <http://www3.ncbi.nlm.nih.gov/Taxonomy/tax.html>.

35

A method of screening for such phytases using PCR is also given, as are general procedures for isolating and purifying these phytase enzyme using recombinant DNA technology.

In a first aspect, the invention relates to an isolated polypeptide having phytase activity and being derived from the phylum Basidiomycota.

5

In a second aspect, the invention relates to an isolated polypeptide having phytase activity and comprising at least one of several consensus sequences.

- 10 In a third aspect, the invention relates to an isolated polypeptide having phytase activity and being encoded by a DNA sequence which hybridizes under medium to high stringency with the product of a PCR reaction using a suitable set of primers derived from alignments disclosed herein and a target sequence,  
15 e.g. a DNA library.

In a fourth aspect, the invention relates to an isolated polypeptide having 6-phytase activity and being derived from a fungus.

20

In a fifth aspect, the invention relates to isolated polypeptides having phytase activity and being homologous to five specific sequences.

- 25 In further aspects, the invention provides cloned DNA sequences encoding the above polypeptides, as well as vectors and host cells comprising these cloned DNA sequences.

Within the scope of the invention, in a still further aspect,  
30 is the use of the phytase of the invention for liberating inorganic phosphate from phytic acid, as well as some more specific uses, and compositions, in particular food and feed preparations and additives comprising the phytase of the invention.

35

Generally, terms and expressions as used herein are to be interpreted as is usual in the art. In cases of doubt, however, the definitions of the present description might be useful.

**GENERAL DEFINITIONS**

By the expression "an isolated polypeptide/enzyme having/exhibiting phytase activity" or "an isolated phytase" is meant any peptide or protein having phytase activity (vide below) and which is essentially free of other non-phytase polypeptides, e.g., at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by SDS-PAGE. Sometimes such polypeptide is alternatively referred to as a "purified" phytase.

The definition of "an isolated polypeptide" also include fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleic acid sequence (or a portion thereof) encoding another polypeptide to a nucleic acid sequence (or a portion thereof) of the present invention. Techniques for producing fusion polypeptides are known in the art, and include, ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator.

The expression "polypeptide or enzyme exhibiting phytase activity" or "phytase" is intended to cover any enzyme capable of effecting the liberation of inorganic phosphate or phosphorous from various myo-inositol phosphates. Examples of such myo-inositol phosphates (phytase substrates) are phytic acid and any salt thereof, e.g. sodium phytate or potassium phytate or mixed salts. Also any stereoisomer of the mono-, di-, tri-, tetra- or penta-phosphates of myo-inositol might serve as a phytase substrate.

In accordance with the above definition, the phytase activity can be determined using any assay in which one of these substrates is used. In the present context (unless otherwise

specified) the phytase activity is determined in the unit of FYT, one FYT being the amount of enzyme that liberates 1 µmol inorganic ortho-phosphate per min. under the following conditions: pH 5.5; temperature 37°C; substrate: sodium phytate (C<sub>6</sub>H<sub>6</sub>O<sub>24</sub>P<sub>6</sub>Na<sub>12</sub>) in a concentration of 0.0050 mol/l. Suitable phytase assays are described in the experimental part.

"Polypeptide homology" or "amino acid homology" is determined as the degree of identity between two sequences. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG version 8 program package (Program Manual for the Wisconsin Package, Version 8, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., 1970), Journal of Molecular Biology, 48, 443-453. Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3.

In the present context a "6-phytase" means a phytase which hydrolyzes first the 6-position in phytic acid or has a preference for these positions (plural is used since this term covers two positions). In particular, more than 50% of the hydrolysis product of the first step is Ins(1,2,3,4,5)P<sub>5</sub> and/or Ins(1,2,3,5,6)P<sub>5</sub>. Preferably these two compounds comprise at least 60%, more preferably at least 70%, still more preferably at least 80%, especially at least 90% and mostly preferred more than 95% of the product of the initial hydrolysis step of PA.

30

The other specificity terms such as e.g. "3-phytase," "(3+6)-phytase" "6D-phytase" and "6L-phytase" are to be interpreted correspondingly, including the same preferred embodiments.

35 The terms "a phytase encoding part of a DNA sequence cloned into a plasmid present in a deposited E. coli strain" and "a phytase encoding part of the corresponding DNA sequence presented in the sequence listing" are presently believed to

be identical, and accordingly they may be used interchangeably.

Primarily, the term "a phytase encoding part" used in  
5 connection with a DNA sequence means that region of the DNA sequence which is translated into a polypeptide sequence having phytase activity. Often this is the region between a first "ATG" start codon ("AUG" codon in mRNA) and a stop codon ("TAA", "TAG" or "TGA") first to follow.

10

However, the polypeptide translated as described above often comprises, in addition to a mature sequence exhibiting phytase activity, an N-terminal signal sequence and/or a pro-peptide sequence. Generally, the signal sequence guides the secretion  
15 of the polypeptide and the pro-peptide guides the folding of the polypeptide. For further information see Egnell, P. et al. Molecular Microbiol. 6(9):1115-19 (1992) or Stryer, L., "Biochemistry" W.H., Freeman and Company/New York, ISBN 0-7167-1920-7. Therefore, the term "phytase encoding part" is  
20 also intended to cover the DNA sequence corresponding to the mature part of the translated polypeptide or to each of such mature parts, if several exist.

Still further, any fragment of such sequence encoding a  
25 polypeptide fragment, which still retains some phytase activity, is to be included in this definition.

An isolated DNA molecule or, alternatively, a "cloned DNA sequence" "a DNA construct," "a DNA segment" or "an isolated  
30 DNA sequence" refers to a DNA molecule or sequence which can be cloned in accordance with standard cloning procedures used in genetic engineering to relocate the DNA segment from its natural location to a different site where it will be replicated. The term refers generally to a nucleic acid  
35 sequence which is essentially free of other nucleic acid sequences, e.g., at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by agarose

gel electrophoresis. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a host cell where multiple copies or clones of the nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

10

The degree of identity or "homology" between two nucleic acid sequences may be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 15 1996, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3.

20

Suitable experimental conditions for determining whether a given DNA or RNA sequence "hybridizes" to a specified nucleotide or oligonucleotide probe involves presoaking of the filter containing the DNA fragments or RNA to examine for hybridization in 5 x SSC (Sodium chloride/Sodium citrate), (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al. 30 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a concentration of 10 ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), <sup>32</sup>P-dCTP-labeled (specific activity > 1 x 35 10<sup>9</sup> cpm/µg) probe for 12 hours at approximately 45°C.

The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at least 55°C (low stringency), at least 60°C (medium stringency), at least 65°C (medium/high

stringency), at at least 70°C (high stringency), or at at least 75°C (very high stringency).

Molecules to which the oligonucleotide probe hybridizes under 5 these conditions are detected using an x-ray film.

It has been found that it is possible to theoretically predict whether or not two given DNA sequences will hybridize under certain specified conditions.

10

Accordingly, as an alternative to the above described experimental method the determination whether or not an analogous DNA sequence will hybridize to the nucleotide probe described above, can be based on a theoretical calculation of 15 the Tm (melting temperature) at which two heterologous DNA sequences with known sequences will hybridize under specified conditions (e.g. with respect to cation concentration and temperature).

20 In order to determine the melting temperature for heterologous DNA sequences ( $T_m(\text{hetero})$ ) it is necessary first to determine the melting temperature ( $T_m(\text{homo})$ ) for homologous DNA sequences.

25 The melting temperature ( $T_m(\text{homo})$ ) between two fully complementary DNA strands (homoduplex formation) may be determined by use of the following formula,

$$T_m(\text{homo}) = 81.5^\circ\text{C} + 16.6(\log M) + 0.41(\%GC) - 0.61(\% \text{ form}) - 30 \frac{500}{L}$$

("Current protocols in Molecular Biology". John Wiley and Sons, 1995), wherein

"M" denotes the molar cation concentration in wash buffer,  
35 "%GC" % Guanine (G) and Cytosine (C) of total number of bases in the DNA sequence,

"% form" % formamid in the wash buffer, and

"L" the length of the DNA sequence.

- The  $T_m$  determined by the above formula is the  $T_m$  of a homoduplex formation ( $T_m(homo)$ ) between two fully complementary DNA sequences. In order to adapt the  $T_m$  value to that of two heterologous DNA sequences, it is assumed that a 1% difference in nucleotide sequence between the two heterologous sequences equals a 1°C decrease in  $T_m$  ("Current protocols in Molecular Biology". John Wiley and Sons, 1995). Therefore, the  $T_m(hetero)$  for the heteroduplex formation is found by subtracting the homology % difference between the analogous sequence in question and the nucleotide probe described above from the  $T_m(homo)$ . The DNA homology percentage to be subtracted is calculated as described herein (vide supra).
- 15 The term "vector" is intended to include such terms/objects as "nucleic acid constructs," "DNA constructs," expression vectors" or "recombinant vectors."

The nucleic acid construct comprises a nucleic acid sequence of the present invention operably linked to one or more control sequences capable of directing the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

25 "Nucleic acid construct" is defined herein as a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature.

The term nucleic acid construct may be synonymous with the term expression cassette when the nucleic acid construct contains all the control sequences required for expression of a coding sequence of the present invention.

The term "coding sequence" as defined herein primarily comprises a sequence which is transcribed into mRNA and translated into a polypeptide of the present invention when

placed under the control of the above mentioned control sequences. The boundaries of the coding sequence are generally determined by a translation start codon ATG at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, DNA, cDNA, and recombinant nucleic acid sequences.

The term "control sequences" is defined herein to include all components which are necessary or advantageous for expression 10 of the coding sequence of the nucleic acid sequence. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, a polyadenylation sequence, a propeptide sequence, a promoter, a signal sequence, 15 and a transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control 20 sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

In the expression vector, the DNA sequence encoding the phytase should be operably connected to a suitable promoter 25 and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins which are either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the 30 phytase, the promoter and the terminator and to insert them into suitable vectors are well known to persons skilled in the art (cf. e.g. Sambrook et al., (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY).

35 The recombinant expression vector may be any vector (e.g., a plasmid or virus) which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleic acid sequence.

- More than one copy of a nucleic acid sequence encoding a polypeptide of the present invention may be inserted into the host cell to amplify expression of the nucleic acid sequence.
- 5 Stable amplification of the nucleic acid sequence can be obtained by integrating at least one additional copy of the sequence into the host cell genome using methods well known in the art and selecting for transformants.
- 10 The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook et al., 1989, *supra*).
- 15 A "host cell" or "recombinant host cell" encompasses any progeny of a parent cell which is not identical to the parent cell due to mutations that occur during replication.

The cell is preferably transformed with a vector comprising a  
20 nucleic acid sequence of the invention followed by integration of the vector into the host chromosome.

"Transformation" means introducing a vector comprising a nucleic acid sequence of the present invention into a host cell  
25 so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is generally considered to be an advantage as the nucleic acid sequence is more likely to be stably maintained in the cell. Integration of the vector into the host chromosome may occur by  
30 homologous or non-homologous recombination as described above.

The host cell may be a unicellular microorganism, e.g., a prokaryote, or a non-unicellular microorganism, e.g., a eukaryote. Examples of a eukaryote cell is a mammalian cell,  
35 an insect cell, a plant cell or a fungal cell. Useful mammalian cells include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, COS cells, or any number of other immortalized cell lines available, e.g., from the American Type Culture Collection.

In a preferred embodiment, the host cell is a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by 5 Hawksworth et al., In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth et al., 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth et al., 1995, *supra*).

10

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*.

15 The present invention also relates to a transgenic plant, plant part, such as a plant seed, or plant cell, which has been transformed with a DNA sequence encoding the phytase of the invention so as to express or produce this enzyme. Also compositions and uses of such plant or plant part are within 20 the scope of the invention, especially its use as feed and food or additives therefore, along the lines of the present use and food/feed claims.

The transgenic plant can be dicotyledonous or 25 monocotyledonous, for short a dicot or a monocot. Of primary interest are such plants which are potential food or feed components and which comprise phytic acid. A normal phytic acid level of feed components is 0.1-100 g/kg, or more usually 0.5-50 g/kg, most usually 0.5-20 g/kg. Examples of monocot 30 plants are grasses, such as meadow grass (blue grass, Poa), forage grass such as festuca, lolium, temperate grass, such as Agrostis, and cereals, e.g. wheat, oats, rye, barley, rice, sorghum and maize (corn).

35 Examples of dicot plants are legumes, such as lupins, pea, bean and soybean, and cruciferous (family Brassicaceae), such as cauliflower, oil seed rape and the closely related model organism *Arabidopsis thaliana*.

Such transgenic plant etc. is capable of degrading its own phytic acid, and accordingly the need for adding such enzymes to food or feed comprising such plants is alleviated.

5 Preferably, the plant or plant part, e.g. the seeds, are ground or milled, and possibly also soaked before being added to the food or feed or before the use, e.g. intake, thereof, with a view to adapting the speed of the enzymatic degradation to the actual use.

10

If desired, the plant produced enzyme can also be recovered from the plant. In certain cases the recovery from the plant is to be preferred with a view to securing a heat stable formulation in a potential subsequent pelleting process.

15

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, tubers etc. But also any plant tissue is included in this definition.

20 Any plant cell, whatever the tissue origin, is included in the definition of plant cells above.

Also included within the scope of the invention are the progeny of such plants, plant parts and plant cells.

25

The skilled man will known how to construct a DNA expression construct for insertion into the plant in question, paying regard i.a. to whether the enzyme should be excreted in a tissue specific way. Of relevance for this evaluation is the 30 stability (pH-stability, degradability by endogenous proteases etc.) of the phytase in the expression compartments of the plant. He will also be able to select appropriate regulatory sequences such as promoter and terminator sequences, and signal or transit sequences if required (Tague et al, Plant, 35 Phys., 86, 506, 1988).

The plant, plant part etc. can be transformed with this DNA construct using any known method. An example of such method is the transformation by a viral or bacterial vector such as

bacterial species of the genus *Agrobacterium* genetically engineered to comprise the gene encoding the phytase of the invention. Also methods of directly introducing the phytase DNA into the plant cell or plant tissue are known in the art, 5 e.g. micro injection and electroporation (Gasser et al, Science, 244, 1293; Potrykus, Bio/Techn. 8, 535, 1990; Shimamoto et al, Nature, 338, 274, 1989).

Following the transformation, the transformants are screened 10 using any method known to the skilled man, following which they are regenerated into whole plants.

These plants etc. as well as their progeny then carry the phytase encoding DNA as a part of their genetic equipment.

15

In general, reference is had to WO 9114782A and WO 9114772A.

*Agrobacterium tumefaciens* mediated gene transfer is the method of choice for generating transgenic dicots (for review Hooykas 20 & Schilperoort, 1992. Plant Mol. Biol. 19: 15-38). Due to host range limitations it is generally not possible to transform monocots with the help of *A. tumefaciens*. Here, other methods have to be employed. The method of choice for generating 25 transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992. Plant J. 2: 275-281; Shimamoto, 1994. Curr. Opin. Biotechnol. 5: 158-162; Vasil et al., 1992. Bio/Technology 10: 667-674).

30 Also other systems for the delivery of free DNA into these plants, including viral vectors (Joshi & Joshi, 1991. FEBS Lett. 281: 1-8), protoplast transformation via polyethylene glycol or electroporation (for review see Potyrkus, 1991. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42: 205-225), 35 microinjection of DNA into mesophyll protoplasts (Crossway et al., 1986. Mol. Gen. Genet. 202: 79-85), and macroinjection of DNA into young floral tillers of cereal plants (de la Pena et al., 1987. Nature 325: 274-276) are preferred methods.

In general, the cDNA or gene encoding the phytase of the invention is placed in an expression cassette (e.g. Pietrzak et al., 1986. Nucleic Acids Res. 14: 5857-5868) consisting of 5 a suitable promoter active in the target plant and a suitable terminator (termination of transcription). This cassette (of course including a suitable selection marker, see below) will be transformed into the plant as such in case of monocots via particle bombardment. In case of dicots the expression 10 cassette is placed first into a suitable vector providing the T-DNA borders and a suitable selection marker which in turn are transformed into *Agrobacterium tumefaciens*. Dicots will be transformed via the *Agrobacterium* harbouring the expression cassette and selection marker flanked by T-DNA following 15 standard protocols (e.g. Akama et al., 1992. Plant Cell Reports 12: 7-11). The transfer of T-DNA from *Agrobacterium* to the Plant cell has been recently reviewed (Zupan & Zambryski, 1995. Plant Physiol. 107: 1041-1047). Vectors for plant transformation via *Agrobacterium* are commercially available or 20 can be obtained from many labs that construct such vectors (e.g. Deblaere et al., 1985. Nucleic Acids Res. 13: 4777-4788; for review see Klee et al., 1987. Annu. Rev. Plant Physiol. 38: 467-486).

25 Available plant promoters: Depending on the process under manipulation, organ- and/or cell-specific expression as well as appropriate developmental and environmental control may be required. For instance, it is desirable to express a phytase cDNA in maize endosperm etc. The most commonly used promoter 30 has been the constitutive 35S-CaMV promoter Franck et al., 1980. Cell 21: 285-294). Expression will be more or less equal throughout the whole plant. This promoter has been used successfully to engineer herbicide- and pathogen-resistant plants (for review see Stitt & Sonnewald, 1995. Annu. Rev. 35 Plant Physiol. Plant Mol. Biol. 46: 341-368). Organ-specific promoters have been reported for storage sink tissues such as seeds, potato tubers, and fruits (Edwards & Coruzzi, 1990. Annu. Rev. Genet. 24: 275-303), and for metabolic sink tissues such as meristems (Ito et al., 1994. Plant Mol. Biol. 24: 863-

878).

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed phytase may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Preferred host cells are a strain of *Fusarium*, *Trichoderma* or *Aspergillus*, in particular a strain of *Fusarium graminearum*, *Fusarium venenatum*, *Fusarium cerealis*, *Fusarium sp.* having the identifying characteristic of *Fusarium* ATCC 20334, as further described in PCT/US/95/07743, *Trichoderma harzianum* or *Trichoderma reesei*, *Aspergillus niger* or *Aspergillus oryzae*.

20

#### BRIEF DESCRIPTION OF THE DRAWINGS

In the detailed description of the invention below, reference is had to the drawings, of which

fig. 1

25 is the nucleotide sequence of the phyA cDNA and the deduced primary struture of PHYA phytase from *Peniophora lycii* (the signal peptide is boxed and the restriction sites used for cDNA cloning are underlined);

30 fig. 2

is the nucleotide sequence of a phytase from *Agrocybe pediades*, as in fig. 1;

fig. 3

35 is the nucleotide sequence of a first phytase, PHYA1, from *Paxillus involutus*, as in fig. 1, except for the box referring to the SignalP V1.1 prediction of the signal peptide;

fig. 4

is the nucleotide sequence of a second phytase, PHYA2, from Paxillus involutus, as in fig. 3;

5

fig. 5

is the nucleotide sequence of a phytase from Trametes pubescens, as in fig. 3;

10 fig. 6

is an alignment of the deduced amino acid sequences of the encoded phytases of figs. 1-5, identical residues in at least three of the sequences being indicated by a grey box;

15

fig. 7

is an alignment of the five phytases of fig. 6 together with five known phytases which all belong to the fungal phylum of Ascomycota, identical residues in at least 20 seven of the sequences being indicated by a grey box;

fig. 8

is a pH-activity curve of the Peniophora phytase;

25 fig. 9

a pH-stability curve thereof;

fig. 10

a temperature-activity curve thereof;

30

fig. 11

a temperature-stability curve thereof;

fig. 12

35 a Differential Scanning Calorimetry (DSC) curve thereof;

figs. 13-14

NMR spectra, stacked plots (up to 24h), showing the product profiling of an Aspergillus niger and the

figs. 30-31

NMR spectra as above, but stacked plots up to 4.5h;

fig. 32-33

5 NMR profiles observed after 20 minutes and 24 hours,  
respectively;

figs. 34-35

10 curves showing concentration versus time of Ins(1,2)P<sub>2</sub>  
and Ins(2)P, respectively;

figs. 36-37

curves showing the release of inorganic phosphate versus  
time from corn at pH 5.5 and pH 3.5, respectively; and

15

fig. 38

the structure of 1D- and 1L-myo-inositol-1-phosphate (P =  
-OPO<sub>3</sub><sup>-2</sup>).

20

#### DEPOSITIONS

Isolates of the strains of *Peniophora lycii*, *Agrocybe pediades*, *Paxillus involutus*, and *Trametes pubescens* from which phytases of the invention were obtained have been deposited  
25 according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Centraalbureau voor Schimmelcultures, P.O. Box 273, 3740 AG Baarn, The Netherlands, (CBS), as follows:

30

Deposit date : 4th of December 1996

Depositor's ref. : NN006113

CBS No. : *Peniophora lycii* CBS No. 686.96

35 Deposit date : 4th of December 1996

Depositor's ref. : NN009289

CBS No. : *Agrocybe pediades* CBS No. 900.96

Peniophora phytase, respectively;

figs. 15-16

NMR spectra as above, but stacked plots up to 4.5h;

5

figs. 17-19

NMR profiles observed after 20 minutes (at pH 5.5), 24 hours (at pH 5.5) and 20 minutes (at pH 3.5), respectively;

10

fig. 20

curves showing concentration versus time of Ins(1,2)P<sub>2</sub> and Ins(2)P, respectively;

15 fig. 21-22

curves showing the release of inorganic phosphate versus time from corn at pH 5.5 and pH 3.5, respectively;

fig. 23

20 is a pH-activity curve of the Agrocybe phytase;

fig. 24

a pH-stability curve thereof;

25 fig. 25

a temperature-activity curve thereof;

fig. 26

a temperature-stability curve thereof;

30

fig. 27

a Differential Scanning Calorimetry (DSC) curve thereof;

figs. 28-29

35 NMR spectra, stacked plots (up to 24h), showing the product profiling of an Aspergillus niger and the Agrocybe phytase, respectively;

Deposit date : 28th of November 1997  
Depositor's ref. : NN005693  
CBS No. : *Paxillus involutus* CBS No. 100231

5 Deposit date : 28th of November 1997  
Depositor's ref. : NN009343  
CBS No. : *Trametes pubescens* CBS No. 100232

Still further, the expression plasmids (shuttle vector) pYES  
10 2.0 comprising the full length cDNA sequences encoding these  
phytases of the invention have been transformed into strains  
of *Escherichia coli* which were deposited according to the  
Budapest Treaty on the International Recognition of the  
Deposit of Microorganisms for the Purposes of Patent Procedure  
15 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen  
GmbH., Mascheroder Weg 1b, D-38124 Braunschweig, Germany,  
(DSM), as follows, respectively (two phytases of *Paxillus*  
*involutus*):

20 Deposit date : 2nd of December 1996  
Depositor's ref. : NN 049282  
DSM No. : *Escherichia coli* DSM No. 11312

Deposit date : 2nd of December 1996  
25 Depositor's ref. : NN 049283  
DSM No. : *Escherichia coli* DSM No. 11313

Deposit date : 12th of November 1997  
Depositor's ref. : NN 049342  
30 DSM No. : *Escherichia coli* DSM No. 11842

Deposit date : 12th of November 1997  
Depositor's ref. : NN 049343  
DSM No. : *Escherichia coli* DSM No. 11843

35 Deposit date : 12th of November 1997  
Depositor's ref. : NN 049344  
DSM No. : *Escherichia coli* DSM No. 11844

#### DETAILED DESCRIPTION OF THE INVENTION

- The phytases of the invention derived from basidiomycetes are 5 preferably derived from the classes *Gasteromycetes*, *Hymenomycetes*, *Urediniomycetes*, *Ustilaginomycetes* or from unclassified *Basidiomycota*, more preferably from the class *Hymenomycetes*.
- 10 The phytases derived from the class *Hymenomycetes* are preferably derived from strains of the orders *Agaricales*, *Aphyllophorales*, *Auriculariales*, *Boletales*, *Cantharellales*, *Ceratobasidiales*, *Dacrymycetales*, *Echinodontiaceae*, *Hericiiales*, *Stereales*, *Thelephorales*, *Tremellales*, *Tulasnellales* or from 15 the class of mitosporic *Hymenomycetes*.

Other preferred orders are *Coriolales*, *Hyphodermatales*, *Schizophyllales*, *Hymenochaetales* and *Phanerochaetales*.

- 20 Below, preferred families of some of these orders are listed, and examples of preferred genera within each family are added in parentheses behind each family.

Preferred families of the order *Aphyllophorales* are 25 *Polyporaceae* (e.g. genus *Trametes*, *Bjerkandera*, *Irpea*, *Oxyporus*, *Trichaptum*, *Daedalea*, *Fomes*), *Coniophoraceae* (e.g. genus *Coniophora*), *Corticaceae* (e.g. genus *Hyphoderma*, *Trechispora*, *Steccherinum*, *Merulius*, *Peniophora*), *Schizophyllaceae* (e.g. genus *Schizophyllum*).

30

Preferred families of the order *Agaricales* are *Bolbitiaceae* (e.g. genus *Agrocybe*, *Conocybe*, *Bolbitius*), *Coprinaceae* (e.g. genus *Coprinus*, *Panaeolus*), *Pluteaceae* (e.g. genus *Volvariella*), *Podaxaceae* (e.g. genus *Podaxis*), *Tricholomataceae* 35 (e.g. genus *Marasmiellus*, *Strobilurus*, *Lyophyllum*, *Cystoderma*, *Merismodes*), *Strophariaceae* (e.g. genus *Stropharia*, *Hypholoma*).

A preferred family of the order *Auriculariales* is *Exidiaceae* (e.g. genus *Exidia*).

A preferred family of the order *Dacrymetales* is *Dacrymycetaceae* (e.g. genus *Femsjonia*).

5 Preferred families of the order *Stereales* are *Hyphodermataceae* (e.g. genus *Hyphodontia*) and *Stereaceae* (e.g. genus *Amylostereum* and *Stereum*).

A preferred family of the order *Boletales* is *Paxillaceae* (e.g. 10 genus *Paxillus* and *Hygrophoropsis*).

A preferred family of the order *Thelephorales* is *Thelephoraceae* (e.g. genus *Typhula*).

15 Some examples of preferred strains of the above genera are *Stropharia cubensis* (in particular ATCC 13966), *Agrocybe pediades* (in particular CBS 900.96), *Bjerkandera adusta* (in particular CBS 580.95), *Trametes zonatella*, *Trametes pubescens* (in particular CBS 100232), *Paxillus involutus* (in particular 20 CBS 100231), *Trametes hirsuta* (in particular DSM 2987), *Peniophora quercina*, *Hyphoderma argillaceum*, *Scizophyllum sp.* (in particular CBS 443.97), *Peniophora lycii* (in particular CBS 686.96), *Amylostereum chailletii*, *Oxyporus sp.* (in particular CBS 422.97). Further examples of preferred strains 25 are listed in Example 5, Table 6.

The phytases of claim 2 have at least one of 14 partial amino acid sequences in common, the so-called consensus sequences, which are entered in the sequence listing as SEQ ID NOS: 1-14.

30

In the sequence listing, the amino acid three-letter-code is used, and some of the amino acids are denoted Xaa, which generally means "any amino acid" interpreted as below. In case of some of these Xaa-positions, however, a note is entered in 35 the sequence listing to the effect that for instance X in position NN means any of two amino acids, cf. below.

In the claims, the amino acid one-letter-code is used in these sequences, and "X" denotes any amino acid including the non-

naturally occurring ones and including any structurally or functionally similar variants thereof. Denotations like "[A/E]" mean any of the amino acids A and E. Accordingly, if in a partial sequence of a given formula two of such multiple choices exist, the number of sequences covered by the formula is  $2^2$ . Likewise, if there are "N" such multiple choices in a given formula, the number of sequences covered by this formula is  $2^N$ .

10 SEQ ID NOS: 1 to 9 are listed in the order of N-terminal to C-terminal end of the polypeptides. SEQ ID NOS: 10 to 14 are the amino acid sequences corresponding to the PCR probes specifically listed in Example 5 (viz. corresponding to the 522-sense and 540-anti-sense; 537-sense; 538-sense; 525-anti-  
15 sense and 539-anti-sense primers, respectively).

In preferred embodiments, the isolated polypeptide comprises at least two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen or all fourteen sequences of SEQ ID  
20 Nos: 1-14, preferably at the positions indicated in claim 3. Claim 3 could also refer to the alignment in Fig. 6.

The sets of amino acid sequences of claim 4 reflect the PCR experiments of Examples 5 and 6 (viz. those primer-sets which  
25 are proposed).

Also some deletions seem to be characteristic for this phytase sub-family. Some regions of specific deletions are listed in claim 5. This claim could also refer to the alignment of  
30 fig. 6.

These partial sequences have been identified i.a. on the basis of the alignment shown in Fig. 7. In this alignment the five phytases at the top, viz. phyA1\_P.involtus, phyA2\_P.involtus,  
35 phyA\_T.pubescens, phyA\_A.pediades and phyA\_P.lycii, are all derived from basidiomycetes and have been cloned as reported in the experimental section hereof. The five phytases listed at the bottom of the alignment are all derived from ascomycetes and their sequences are known from the prior art

mentioned previously. Please refer to Example 4 hereof for further details and explanations with respect to the alignments, and please refer to Example 5 for one way of carrying out screening for phytases of this sub-family.

5 Examples 8-18 describe the purification and characterization of five phytases of the invention, viz. the phytases of *Peniophora lycii*, *Agrocybe pediades*, *Paxillus involutus* and *Trametes pubescens*.

10 Claims 6-8 are related to the experiments of Examples 5, 6 and 7. The conditions corresponding to the term "medium to high stringency" are found in the general definition part above, viz. the washing conditions are 2 X SSC and a temperature of 65°C. Preferably, the washing temperature is 65°C or even 15 higher, e.g. 70, 75, 80 or even 85°C, coresponding to high stringency, very high stringency or exceptionally high stringency.

20 Preferably, the PCR reaction is performed with a template or a target sequence, e.g. a nucleotide sequence, which can be e.g. genomic DNA or cDNA. However, for instance mRNA can also be used as a template. Genomic DNA need not be isolated, the PCR reaction can also be conducted directly on for instance fungal mycelium.

25

Alternatively, the PCR reaction is performed with the wild-type gene of any PE variant thereof. In particular, at least one PCR band is obtained using at least one primer set on the wild type gene.

30

Some specific primers for the PCR reaction are suggested in the experimental part. However, the skilled man is certainly able to propose also other specific primers from the alignment at fig. 6 (basidiomycete phytases) which primers seem 35 characteristic for basidiomycete phytases, i.e. he has to consider also the alignment at fig. 7 (showing basidiomycete phytases as well as known ascomycete phytases). Therefore, claim 6 refers in general to such primers, as the skilled man would suggest be specific for basidiomycete phytases, based on

his common general knowledge and the alignments at figs. 6 and 7.

As regards 6-phytases, the invention relates to such phytases derived from any fungus. "Fungal" is defined as described above and this term includes i.a. basidiomycetes. How to interpret the specificity is explained in the general definitions part hereof. It is noted, that in the present context, the concept of "a 6-phytase" means any of a D6-, L6- or D/L-6-phytase. Surprisingly, it has turned up that such fungal 6-phytases are of a superior performance as compared to known fungal 3-phytases, reference being had to Examples 10-12 hereof revealing the Peniophora phytase as a 6-phytase and of a highly superior performance as compared to the known Aspergillus phytases. In particular, e.g. the initial rate of hydrolysis of PA is increased and a very plausible explanation of this fact could be that this phytase is a 6-phytase, since these positions (4- and 6- in PA) are less sterically hindered than any of the other positions.

20

Preferably, the fungal 6-phytase is a basidiomycete phytase, still more preferably of the class, sub-class, orders, genera and strains as described at the beginning of this section headed "Detailed description of the invention." In another preferred embodiment the phytase is a D6-phytase. In another preferred embodiment the phytase is a L6-phytase.

As is apparent from claims 32-36 this application also relates to e.g. the use of a fungal 6-phytase in feed and food, compositions comprising such fungal 6-phytase, in particular food and feed additives, and the use of such fungal 6-phytase for liberating inorganic phosphate from phytic acid or phytate.

In a preferred embodiment of claims 1-8, the phytase is a (3+6)-phytase, viz. any of a D3-/D6-; L3-/L6-; D3-/L6-; L3-/D6-phytase, reference being had in particular to Examples 15-17 herein regarding the Agrocybe phytase which is also of superior performance as compared to the known Aspergillus phytase.

Preferably, the (3+6)-phytase is a basidiomycete phytase, still more preferably of the class, sub-class, orders, genera and strains as described at the beginning of this section headed  
5 "Detailed description of the invention."

Still more preferably, the (3+6)-phytase has a slight preference for one of these positions, in particular the 6-position.

10

The phytases of claims 11, 14, 17, 19 and 21 are all more than 50% homologous to the isolated phytases of *Agrocybe pediades*, *Peniophora lycii*, *Paxillus involutus* (phyA1 and phyA2) and *Trametes pubescens*, respectively, corresponding to SEQ ID Nos:  
15 22, 24, 26, 28 and 30, respectively (or the mature polypeptides thereof or any fragment thereof still retaining phytase activity). For a definition of "homologous", please refer to the section headed "General definitions." The homology to known phytases appear i.a. from Table 1 in Example  
20 4. Preferably, the number of amino acids in the fragments referred to above is at least 50%, more preferably at least 60%, still more preferably at least 70%, even more preferably at least 80%, in particular at least 90% of the number of amino acids of the sequences listed in the sequence listing.  
25 This is so also for any polypeptide fragment disclosed herein.

Preferably, all amino acid homologies of the present application are at least 55%, or at least 60%, or at least 65%, especially at least 70%. Preferably, the degree of  
30 homology is at least 80%, more preferably at least 90%, still more preferably at least 95%, especially at least 97%.

Claim 12 relates to certain fragments of the amino acid sequence of SEQ ID NO 22 derived from *Agrocybe*, these  
35 fragments, however, still exhibiting phytase activity. As described in more detail in the experimental part below (Examples 13-15), when expressed in yeast approximately 80% of the *Agrocybe* phytase enzyme has the N-terminal amino acid of Val (amino acid no. 27 in SEQ ID NO 22), whereas approximately

20% has the N-terminal amino acid of Thr (amino acid no. 25 in SEQ ID NO 22). When expressed in *Aspergillus* approximately 2/3 has the N-terminal amino acid of Phe (amino acid no. 31 in SEQ ID NO 22), whereas approximately 1/3 has the N-terminal amino acid of Gln (amino acid no. 28 in SEQ ID NO 22). Accordingly, there are at least these four possible mature amino acid sequences.

Analogously, claim 15 relates to a fragment of the amino acid sequence of SEQ ID NO 24 derived from *Peniophora*, this fragment, however, still exhibiting phytase activity. As described in more detail in the experimental part below, when expressed in *Aspergillus*, the *Peniophora* phytase has an N-terminal amino acid sequence of Leu-Pro-Ile-Pro-Ala-Gln-Asn- (amino acids no. 31-37 in SEQ ID NO 24). Accordingly the sequence of amino acids nos. 31-439 of SEQ ID No 24 is presently believed to be a mature phytase sequence.

Claims 13, 16, 18, 20 and 22 are drawn to phytases homologous to the isolated phytases of *Agrocybe pediades*, *Peniophora lycii*, *Paxillus involutus* (phyA1 and phyA2) and *Trametes pubescens*, respectively.

These phytases are here defined as being encoded by a phytase encoding part of

- i) the DNA sequences listed in the sequence listing as SEQ ID Nos: 21, 23, 25, 27 and 29, respectively (phyA1 and phyA2 of *Paxillus involutus*); or
- ii) the DNA sequences cloned into plasmid pYES 2.0 present in *E. coli* DSM 11313, 11312, 11842, 11843 and 11844, respectively; or analogues or derivatives thereof which are at least 50% homologous thereto.

For the definition of a "phytase encoding part" please refer to the general definitions section.

The five DNA sequences are obtainable directly from the deposited parent strains or from the deposited *E. coli* strains

by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

5 Claims 23-26 relate to nucleotide sequences encoding the phytases of the invention, in particular to DNA molecules.

The DNA molecule of claim 24 comprises at least one of the specific primers suggested herein. In preferred embodiments, 10 it comprises at least two, three, four, five or six of these sequences.

The DNA molecule of claim 25 is defined by encoding a phytase, and being selected from:

- 15 (a) the specific nucleotide sequences of the *Agrocybe*, *Peniophora*, *Paxillus* and *Trametes* phytases (*phyA1* and *phyA2* of *Paxillus*), e.g. DNA as shown in the sequence listings having the SEQ ID nos indicated (or their complementary strands);
- 20 (b) the same sequences as under (a) but expressed via the deposited plasmid clones;
- (c) sequences which are at least 55% homologous to these sequences;
- (d) sequences hybridizing under low stringency with the 25 sequences of (a) or (b);
- (e) sequences which do not hybridize because of the degeneracy of the genetic code but encode the specific polypeptides or phytase active fragments thereof.
- 30 For a definition of "hybridization," please refer to the section headed "General definitions," which also lists some preferred hybridization conditions.

With respect to the homology part in feature (c), the degree of 35 homology to the nucleic acid sequence set forth under heading (a) and (b) is at least about 55%, (still encoding an polypeptide exhibiting phytase activity). In particular, the homology is at least 60%, or at least 65%, especially at least 70%, preferably at least about 80%, more preferably at least

about 90%, even more preferably at least about 95%, and most preferably at least about 97%. In particular, the degree of homology is based on a comparison with the entire sequences listed or their complementary strand or any of the sub-  
5 sequences thereof corresponding to a "mature" phytase.

The homology of the DNA of selected phytases of the invention to known phytases appear i.a. from Table 1 in Example 4.

- 10 Nucleotide claim 26 is related to polypeptide claims 6-8, and corresponding preferred embodiments mentioned with respect to these claims are hereby incorporated also with respect to this DNA claim (reference to Examples 5, 6 and 7; "medium to high stringency" means the washing conditions are 2 X SSC and a  
15 temperature of 65°C, preferably 65°C or even higher, e.g. 70, 75, 80 or even 85°C; the PCR reaction is performed with a target nucleotide sequence, e.g. DNA, for instance genomic DNA or cDNA (or mRNA); wild-type gene of any PE variant thereof).
- 20 The DNA sequences of the invention can also be cloned by any general method involving
- cloning, in suitable vectors, a cDNA library from any organism expected to produce the phytase of interest, transforming suitable yeast host cells with said vectors,
  - 25 • culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the cDNA library,  
screening for positive clones by determining any phytase activity of the enzyme produced by such clones, and
  - 30 isolating the enzyme encoding DNA from such clones.

A general isolation method has been disclosed in WO 93/11249 and WO 94/14953, the contents of which are hereby incorporated by reference. A detailed description of the screening methods  
35 is given in the experimental part.

Claim 27 relates to a primer, probe, oligonucleotide molecule/DNA molecule which can be derived from the alignment

of fig. 6. Only primers specific or unique for the novel phytases of the invention are of course included herein, viz. one has to consider also the alignment at fig. 7 (cf. remarks to claims 6-8 above).

5

A method of identifying further phytase producing cells, in particular microorganisms, is disclosed in claim 31. In particular, this is also a method of selecting or screening for phytase producing microorganisms. The concept of "cell" is here generally to be defined as the term "host cell" in the general definitions part hereof). Preferred cells are microorganism cells, in particular fungal cells, preferably of the phylum Basidiomycota. More preferred basidiomycete cells are listed in the very beginning of the detailed description 15 of the invention.

Any source, in particular any microorganism, can be selected to provide a template or a target sequence, usually in the form of genomic DNA, cDNA or mRNA.

20

General references to the PCR reaction, including standard reaction conditions, are found in the experimental part. As regards the selection of suitable primers, reference is had to the remarks under claims 6-8 above.

25

Preferably, it has to be verified that the amplified PCR fragment derived from the template is specific.

Some examples of suitable verification procedures are:

- 30 a) running an electrophoresis in agarose gels revealing the existence of a PCR band corresponding to the amplified PCR fragment; and, if desired, also
- b) controlling that the size of the amplified PCR-fragment is as expected; and, if desired, also
- 35 c) isolating and sequencing the PCR fragment or band to show a high degree of homology to the parent sequences, from which the primers were derived.

Ad b): The potential presence of introns, (an example: 50 bases out of 300), is one of several reasons for allowing a deviation from exact size match. Preferably, the size of the amplified PCR-fragment (including introns) as measured for instance by the number of bases is within the ranges of 50-150%, 60-140%, 70-130%, 80-120%, 85-115%, 90-110%, 95-105% of the number of bases/nucleotides inbetween the primers in the parent sequences (fig. 6), from which the primers were derived. In another preferred embodiment (excluding introns), the size of the amplified PCR-fragment is within the range of 80-120%, 85-115%, 90-110%, 95-105% of the number of bases inbetween the primers in the parent sequences (fig. 6), from which the primers were derived.

15 Ad c): Preferably the degree of homology is more than 50, 60, 70, 75, 80, 85, 90, 95% homology (determined as described above). Alternatively, it is checked if the amplified PCR-fragment comprises at least one of the conserved regions inbetween the primers used, said conserved regions being shown 20 in grey shading at fig. 6. Preferably, the fragment comprises at least two, three, four, five etc. or all of such conserved regions. Another way of checking homology is by checking presence of areas of deletions characteristic for the parent sequences of fig. 6. A further way of checking homology is 25 checking characteristic distances between conserved regions, vide e.g. claim 5.

Claim 34 relates to a process for preparing the phytase polypeptide, said process logically following from this 30 screening method of claim 31.

Steps a)+d) of claim 34 relate to the preparation of the phytase from the wild-type cell, in particular microorganism strain.

35

Steps b)+d) relate to the cloning of the entire phytase encoding gene from the under a) identified phytase producing cell, in particular microorganism, and transferring this gene into a heterologous or homologous host cell using any general

recombinant DNA technic, e.g. as hereinbefore described and referring generally to e.g. Maniatis (cited above).

Steps c)+d) relate to the use of the amplified PCR fragment as  
5 a hybridization probe to identify and isolate phytase encoding  
DNA molecules (phytase encoding genes or phytase encoding  
parts of genes). Such DNA molecules may be isolated from any  
source (target sequence, template) which comprises  
polynucleotides, such as genomic DNA, cDNA, mRNA.

10

Hybridization experiments and in particular hybridization conditions, including preferred conditions, and isolation procedures are as generally hereinbefore described.

15 Once isolated, the DNA molecule is transferred to a host cell, as is also generally hereinbefore described.

Finally, the invention also relates generally to the use of the polypeptide according to any of claims 1-22 for liberating  
20 (or catalyzing the liberation of) phosphorous from any phytase substrate, in particular inorganic phosphate from phytate or phytic acid; alternatively for converting phytate to inorganic phosphate and (myo-inositol and/or mono-, di-, tri-, tetra-, penta-phosphate esters thereof). This claim encompasses any  
25 process wherein the phytase of the invention exerts its phytase activity as previously defined.

More specific uses according to the invention are in human food or animal feed preparations or in additives for such  
30 preparations, wherein the phytase i.a. serves the purposes of  
(i) reducing the phytate level of manure,  
(ii) improving the digestibility, promoting the growth, or improving the food and feed utilization or its conversion efficiency, i.a. by making available proteins, which  
35 would otherwise have been bound by phytate,  
(iii) preventing malnutrition or diseases such as anemia caused by essential ions and phosphate lacking, i.e. improving the bioavailability of minerals or increasing the absorption thereof, eliminating the need for adding

supplemental phosphate and ions etc.

In particular, the phytases of the invention can also be used in chicken food to improve egg shell quality (reduction of losses due to breaking), cf. e.g. The Merck Veterinary Manual, (Seventh edition, Merck & CO., Inc., Rahway, N.J., U.S.A., 1991; p.1268); Jeroch et al; Bodenkultur Vol. 45, No. 4 pp. 361-368 (1994); Poultry Science, Vol. 75, No. 1 pp. 62-68 (1996); Canadian Journal of Animal Science Vol. 75 , No. 3 pp. 10 439-444 (1995 ); Poultry Science Vol. 74 , No. 5 pp. 784-787 (1995) and Poultry Science Vol. 73 , No. 10 pp. 1590-1596 (1994).

A "feed" and a "food," respectively, means any natural or 15 artificial diet, meal or the like or components of such meals intended or suitable for being eaten, taken in, digested, by an animal and a human being, respectively.

The phytase may exert its effect *in vitro* or *in vivo*, i.e. 20 before intake or in the stomach of the individual, respectively. Also a combined action is possible.

A phytase composition according to the invention always comprises at least one phytase of the invention.

25

Generally, phytase compositions are liquid or dry.

Liquid compositions need not contain anything more than the phytase enzyme, preferably in a highly purified form. Usually, 30 however, a stabilizer such as glycerol, sorbitol or mono propylen glycol is also added. The liquid composition may also comprise other additives, such as salts, sugars, preservatives, pH-adjusting agents, proteins, phytate (a phytase substrate). Typical liquid compositions are aqueous or 35 oil-based slurries. The liquid compositions can be added to a food or feed after an optional pelleting thereof.

Dry compositions may be spraydried compositions, in which case the composition need not contain anything more than the enzyme

in a dry form. Usually, however, dry compositions are so-called granulates which may readily be mixed with e.g. food or feed components, or more preferably, form a component of a pre-mix. The particle size of the enzyme granulates preferably is 5 compatible with that of the other components of the mixture. This provides a safe and convenient mean of incorporating enzymes into e.g. animal feed.

Agglomeration granulates are prepared using agglomeration 10 technique in a high shear mixer (e.g. Lödige) during which a filler material and the enzyme are co-agglomerated to form granules. Absorption granulates are prepared by having cores of a carrier material to absorb/be coated by the enzyme.

15 Typical filler materials are salts such as disodium sulphate. Other fillers are kaolin, talc, magnesium aluminium silicate and cellulose fibres. Optionally, binders such as dextrins are also included in agglomeration granulates.

20 Typical carrier materials are starch, e.g. in the form of cassava, corn, potato, rice and wheat. Salts may also be used.

Optionally, the granulates are coated with a coating mixture. Such mixture comprises coating agents, preferably hydrophobic 25 coating agents, such as hydrogenated palm oil and beef tallow, and if desired other additives, such as calcium carbonate or kaolin.

Additionally, phytase compositions may contain other 30 substituents such as colouring agents, aroma compounds, stabilizers, vitamins, minerals, other feed or food enhancing enzymes etc. This is so in particular for the so-called pre-mixes.

35 A "food or feed additive" is an essentially pure compound or a multi component composition intended for or suitable for being added to food or feed. In particular it is a substance which by its intended use is becoming a component of a food or feed product or affects any characteristics of a food or feed

product. It is composed as indicated for phytase compositions above. A typical additive usually comprises one or more compounds such as vitamins, minerals or feed enhancing enzymes and suitable carriers and/or excipients.

5

In a preferred embodiment, the phytase compositions of the invention additionally comprises an effective amount of one or more feed enhancing enzymes, in particular feed enhancing enzymes selected from the group consisting of  $\alpha$ -galactosidases,

10  $\beta$ -galactosidases, in particular lactases, other phytases,  $\beta$ -glucanases, in particular endo- $\beta$ -1,4-glucanases and endo- $\beta$ -1,3(4)-glucanases, cellulases, xylosidases, galactanases, in particular arabinogalactan endo-1,4- $\beta$ -galactosidases and arabinogalactan endo-1,3- $\beta$ -galactosidases, endoglucanases, in  
15 particular endo-1,2- $\beta$ -glucanase, endo-1,3- $\alpha$ -glucanase, and endo-1,3- $\beta$ -glucanase, pectin degrading enzymes, in particular pectinases, pectinesterases, pectin lyases, polygalacturonases, arabinanases, rhamnogalacturonases, rhamnogalacturonan acetyl esterases, rhamnogalacturonan- $\alpha$ -rhamnosidase, pectate lyases,  
20 and  $\alpha$ -galacturonisidases, mannanases,  $\beta$ -mannosidases, mannan acetyl esterases, xylan acetyl esterases, proteases, xylanases, arabinoxylanases and lipolytic enzymes such as lipases, phospholipases and cutinases.

25 The animal feed additive of the invention is supplemented to the mono-gastric animal before or simultaneously with the diet. Preferably, the animal feed additive of the invention is supplemented to the mono-gastric animal simultaneously with the diet. In a more preferred embodiment, the animal feed additive  
30 is added to the diet in the form of a granulate or a stabilized liquid.

An effective amount of phytase in food or feed is from about 10-20.000; preferably from about 10 to 15.000, more preferably  
35 from about 10 to 10.000, in particular from about 100 to 5.000, especially from about 100 to about 2.000 FYT/kg feed or food.

Examples of other specific uses of the phytase of the invention is in soy processing and in the manufacture of inositol or derivatives thereof.

5

The invention also relates to a method for reducing phytate levels in animal manure, wherein the animal is fed a feed comprising an effective amount of the phytase of the invention. As stated in the beginning of the present application one 10 important effect thereof is to reduce the phosphate pollution of the environment.

Also within the scope of the invention is the use of a phytase of the invention during the preparation of food or feed 15 preparations or additives, i.e. the phytase exerts its phytase activity during the manufacture only and is not active in the final food or feed product. This aspect is relevant for instance in dough making and baking.

20 The invention also relates to substantially pure biological cultures of the deposited microorganisms and to strains comprising, as a part of their genetic equipment, a DNA sequence encoding a phytase of the invention. Included within the definition of a substantially pure biological culture is 25 any mutant of said strains having retained the phytase encoding capability.

The invention is described in further detail in the following examples which are not in any way intended to limit the scope 30 of the invention.

#### **EXAMPLES**

##### Materials and Methods

###### Media:

35 *Phytate replication plates:*

Add to 200ml of melted SC agar

20ml 20% galactose

800µl 5% threonine

25ml solution A

25ml solution B

200 $\mu$ l Trace element solution (DSM Catalogue 141)

5 *Solution A:*

6g CaCl<sub>2</sub>, 2H<sub>2</sub>O

8g MgCl<sub>2</sub>, 6H<sub>2</sub>O

add ddH<sub>2</sub>O to 1l

pH = 6.5

10

*Solution B:*

35.12g Na-phytate

add H<sub>2</sub>O to 1l

pH = 6.5

15

*Medium A:*

Yeast Nitrogen Base w/o Amino acids (Difco0919)	7.5 g/l
---	---------

Succinic acid (Merck 822260)	11.3 g/l
------------------------------	----------

NaOH (Merck 6498)	6.8 g/l
-------------------	---------

20 Casamino acid w/o vitamin (Difco 0288)	5.6 g/l
---	---------

tryptophan (Merck 8374)	0.1 g/l
-------------------------	---------

Threonine	1.0 g/l
-----------	---------

Na-phytate (35.12 g/l pH 6.5)	125 ml/l
-------------------------------	----------

Galactose	20.0 g/l
-----------	----------

25 Trace metal (DSM 141)	1.0 ml/l
--------------------------	----------

ad 1l with H <sub>2</sub> O	
-----------------------------	--

*Trace metal solution:*

Nitriilotriacetic acid	1.50 g
------------------------	--------

30 MgSO <sub>4</sub> , 7 H <sub>2</sub> O	3.00 g
---	--------

MnSO <sub>4</sub> .2H <sub>2</sub> O	0.50 g
--------------------------------------	--------

NaCl	1.00 g
------	--------

FeSO <sub>4</sub> , 7H <sub>2</sub> O	0.10 g
---------------------------------------	--------

CoSO <sub>4</sub> .7H <sub>2</sub> O	0.18 g
--------------------------------------	--------

35 CaCl <sub>2</sub> , 2H <sub>2</sub> O	0.10 g
--	--------

ZnSO <sub>4</sub> , 7H <sub>2</sub> O	0.18 g
---------------------------------------	--------

CuSO <sub>4</sub> , 5H <sub>2</sub> O	0.01 g
---------------------------------------	--------

KAl(SO <sub>4</sub> ) <sub>2</sub> , 12H <sub>2</sub> O	0.02 g
---	--------

$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.01 g
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	0.025 g
$\text{Na}_2\text{Se}_3\text{O} \cdot 5\text{H}_2\text{O}$	0.30 g
Distilled water	1 l
5 pH 7.0	

First dissolve nitrilotriacetic acid and adjust pH to 6.5 with KOH, then add minerals. Final pH 7.0 (with KOH).

10 *Medium B:*

Similar to medium A except for glucose is added as a C-source instead of galactose.

*YPD:*

15 10 g yeast extract, 20 g peptone,  $\text{H}_2\text{O}$  to 900 ml. Autoclaved, 100 ml 20% glucose (sterile filtered) added.

*YPM:*

10 g yeast extract, 20 g peptone,  $\text{H}_2\text{O}$  to 900 ml. Autoclaved,  
20 100 ml 20% maltodextrin (sterile filtered) added.

*10 x Basal salt:*

75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH,  $\text{H}_2\text{O}$  ad 1000 ml, sterile filtered.

25

*SC-URA:*

100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan,  $\text{H}_2\text{O}$  ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

30

*SC-agar:*

SC-URA, 20 g/l agar added.

*SC-variant agar:*

35 20 g agar, 20 ml 10 x Basal salt,  $\text{H}_2\text{O}$  ad 900 ml, autoclaved

Phytase activity assay

The phytase activity can be measured using the following assay:

10 µl diluted enzyme samples (diluted in 0.1 M sodium acetate,  
5 0.01 % Tween20, pH 5.5) were added into 250 µl 5 mM sodium  
phytate (Sigma) in 0.1 M sodium acetate, 0.01 % Tween20, pH  
5.5 (pH adjusted after dissolving the sodium phytate; the  
substrate was preheated) and incubated for 30 minutes at 37°C.  
The reaction was stopped by adding 250 µl 10 % TCA and free  
10 phosphate was measured by adding 500 µl 7.3 g FeSO<sub>4</sub> in 100 ml  
molybdate reagent (2.5 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O in 8 ml H<sub>2</sub>SO<sub>4</sub>, diluted  
to 250 ml). The absorbance at 750 nm was measured on 200 µl  
samples in 96 well microtiter plates. Substrate and enzyme  
blanks were included. A phosphate standard curve was also  
15 included (0-2 mM phosphate). 1 FYT equals the amount of enzyme  
that releases 1 µmol phosphate/min at the given conditions.

General molecular biology methods

Unless otherwise mentioned the DNA manipulations and  
20 transformations were performed using standard methods of  
molecular biology (Sambrook et al. (1989) Molecular cloning: A  
laboratory manual, Cold Spring Harbor lab., Cold Spring  
Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in  
Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R.,  
25 and Cutting, S. M. (eds.) "Molecular Biological Methods for  
Bacillus". John Wiley and Sons, 1990).

Unless otherwise specified all enzymes for DNA manipulations,  
such as e.g. restriction endonucleases, ligases etc., were  
30 obtained from New England Biolabs, Inc. The enzymes were used  
according to the specifications of the suppliers.

**EXAMPLE 1**

**Cloning and expression of a phytase from *Peniophora lycii* CBS  
35 No. 686.96**

Deposited organisms:

*Peniophora lycii* CBS No. 686.96 comprises a phytase encoding  
DNA sequence of the invention.

*Escherichia coli* DSM NO 11312 contains the plasmid comprising the full length cDNA sequence, coding for a phytase of the invention, in the shuttle vector pYES 2.0.

5 Other strains:

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124 (van den Hazel, H.B; Kielland-Brandt, M.C.; Winther, J.R. in Eur. J. Biochem., 207, 277-283, 1992; (MAT<sub>a</sub>; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1:: LEU2; 10 cir+).

*E. coli* strain: DH10B (Life Technologies)

Plasmids:

15 The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

pYES 2.0 (Invitrogen)

20

pA2phy2 (See example 1)

Expression cloning in yeast

Expression cloning in yeast was done as described by H. 25 Dalboege et al. (H. Dalboege et al Mol. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953), which are hereby incorporated as reference. All individual steps of Extraction of total RNA, cDNA synthesis, Mung bean nuclease treatment, Blunt-ending with T4 DNA polymerase, and Construction of 30 libraries was done according to the references mentioned above.

Fermentation procedure of *Peniophora lycii* CBS No. 686.96 for mRNA isolation:

35 *Peniophora lycii* CBS 686.96 was inoculated from a plate with outgrown mycelium into a shake flask containing 100 ml medium B (soya 30 g/l, malto dextrin 15 g/l, bacto peptone 5 g/l, pluronic 0.2 g/l). The culture was incubated stationary at

26°C for 15 days. The resulting culture broth was filtered through miracloth and the mycelium was frozen down in liquid nitrogen.

5 mRNA was isolated from mycelium from this culture as described in (H. Dalboege et al Mol. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953).

Extraction of total RNA was performed with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion, and isolation of poly(A)<sup>+</sup>RNA was carried out by oligo(dT)-cellulose affinity chromatography using the procedures described in WO 94/14953.

15 cDNA synthesis:

Double-stranded cDNA was synthesized from 5 mg poly(A)<sup>+</sup> RNA by the RNase H method (Gubler and Hoffman (1983) Gene 25:263-269, Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). The poly(A)<sup>+</sup> RNA (5 µg in 5 µl of DEPC-treated water) was heated at 70°C for 8 min. in a pre-siliconized, RNase-free Eppendorph tube, quenched on ice and combined in a final volume of 50 µl with reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, Bethesda Research Laboratories) containing 1 mM dATP, dGTP and dTTP and 0.5 mM 5-methyl-dCTP (Pharmacia), 40 units human placental ribonuclease inhibitor (RNasin, Promega), 1.45 µg of oligo(dT)<sub>18</sub>-Not I primer (Pharmacia) and 1000 units SuperScript II RNase H reverse transcriptase (Bethesda Research Laboratories). First-strand cDNA was synthesized by incubating the reaction mixture at 45°C for 1 hour. After synthesis, the mRNA:cDNA hybrid mixture was gelfiltrated through a MicroSpin S-400 HR (Pharmacia) spin column according to the manufacturer's instructions.

35

After the gelfiltration, the hybrids were diluted in 250 µl second strand buffer (20 mM Tris-Cl, pH 7.4, 90 mM KCl, 4.6 mM MgCl<sub>2</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.16 mM bNAD+) containing 200 µl of each

dNTP, 60 units *E. coli* DNA polymerase I (Pharmacia), 5.25 units RNase H (Promega) and 15 units *E. coli* DNA ligase (Boehringer Mannheim). Second strand cDNA synthesis was performed by incubating the reaction tube at 16°C for 2 hours 5 and additional 15 min. at 25°C. The reaction was stopped by addition of EDTA to a final concentration of 20 mM followed by phenol and chloroform extractions.

Mung bean nuclease treatment:

- 10 The double-stranded cDNA was precipitated at -20°C for 12 hours by addition of 2 vols 96% EtOH, 0.2 vol 10 M NH<sub>4</sub>Ac, recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 µl Mung bean nuclease buffer (30 mM NaAc, pH 4.6, 300 mM NaCl, 1 mM ZnSO<sub>4</sub>, 0.35 mM DTT, 2% glycerol) 15 containing 25 units Mung bean nuclease (Pharmacia). The single-stranded hair-pin DNA was clipped by incubating the reaction at 30°C for 30 min., followed by addition of 70 µl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction and precipitation with 2 vols of 96% EtOH and 0.1 vol 3 M NaAc, pH 20 5.2 on ice for 30 min.

Blunt-ending with T4 DNA polymerase:

The double-stranded cDNAs were recovered by centrifugation and blunt-ended in 30 ml T4 DNA polymerase buffer (20 mM Tris-25 acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 0.5 mM of each dNTP and 5 units T4 DNA polymerase (New England Biolabs) by incubating the reaction mixture at 16°C for 1 hour. The reaction was stopped by addition of EDTA to a final concentration of 20 mM, followed by phenol and chloroform 30 extractions, and precipitation for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Adaptor ligation, Not I digestion and size selection:

After the fill-in reaction the cDNAs were recovered by 35 centrifugation, washed in 70% EtOH and dried. The cDNA pellet was resuspended in 25 µl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM ATP) containing 2.5 µg non-palindromic BstXI adaptors (Invitrogen) and 30 units T4 ligase (Promega) and incubated at 16°C for 12 hours. The reaction was

stopped by heating at 65°C for 20 min. and then cooling on ice for 5 min. The adapted cDNA was digested with Not I restriction enzyme by addition of 20 µl water, 5 µl 10x Not I restriction enzyme buffer (New England Biolabs) and 50 units 5 Not I (New England Biolabs), followed by incubation for 2.5 hours at 37°C. The reaction was stopped by heating at 65°C for 10 min. The cDNAs were size-fractionated by gel electrophoresis on a 0.8% SeaPlaque GTG low melting temperature agarose gel (FMC) in 1x TBE to separate unligated 10 adaptors and small cDNAs. The cDNA was size-selected with a cut-off at 0.7 kb and rescued from the gel by use of b-Agarase (New England Biolabs) according to the manufacturer's instructions and precipitated for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

15

#### Construction of libraries:

The directional, size-selected cDNA was recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 µl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. The cDNAs were 20 desalted by gelfiltration through a MicroSpin S-300 HR (Pharmacia) spin column according to the manufacturer's instructions. Three test ligations were carried out in 10 µl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM ATP) containing 5 µl double-stranded cDNA (reaction 25 tubes #1 and #2), 15 units T4 ligase (Promega) and 30 ng (tube #1), 40 ng (tube #2) and 40 ng (tube #3, the vector background control) of BstXI-NotI cleaved pYES 2.0 vector. The ligation reactions were performed by incubation at 16°C for 12 hours, heating at 70°C for 20 min. and addition of 10 µl water to 30 each tube. 1 µl of each ligation mixture was electroporated into 40 µl electrocompetent *E. coli* DH10B cells (Bethesda research Laboratories) as described (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). Using the optimal conditions a 35 library was established in *E. coli* consisting of pools. Each pool was made by spreading transformed *E. coli* on LB+ampicillin agar plates giving 15.000-30.000 colonies/plate after incubation at 37°C for 24 hours. 20 ml LB+ampicillin was added to the plate and the cells were suspended herein. The

cell suspension was shaked in a 50 ml tube for 1 hour at 37°C. Plasmid DNA was isolated from the cells according to the manufacturer's instructions using QIAGEN plasmid kit and stored at -20°C.

5

1 µl aliquots of purified plasmid DNA (100 ng/ml) from individual pools were transformed into *S. cerevisiae* W3124 by electroporation (Becker and Guarante (1991) Methods Enzymol. 194:182-187) and the transformants were plated on SC agar containing 2% glucose and incubated at 30°C.

Identification of positive colonies:

After 3-5 days of growth, the agar plates were replica plated onto a set of the phytate replication plates, and incubated 15 for 3-5 days at 30°C. 1% LSB-agarose (Sigma) containing 0.2M CaCl<sub>2</sub> is poured over the plates and after 1-4 days the phytase positive colonies are identified as colonies surrounded by a clearing zone.

20 Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the phytase-producing colonies identified.

25 Isolation of a cDNA gene for expression in *Aspergillus*:

A phytase-producing yeast colony was inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

30

DNA was isolated according to WO 94/14953 and dissolved in 50 ml water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme 35 analysis.

The cDNA insert was excised using the restriction enzymes Hind III and Xba I and ligated into the *Aspergillus* expression vector pHD414 resulting in the plasmid pA2phy2.

The cDNA inset of Qiagen purified plasmid DNA of pA2phy2 (Qiagen, USA) was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) and synthetic 5 oligonucleotide primers using an Applied Biosystems ABI PRISM™ 377 DNA Sequencer according to the manufacturers instructions.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*

Protoplasts are prepared as described in WO 95/02043, p. 16, 10 line 21 - page 17, line 12.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM CaCl<sub>2</sub>). Protoplasts are mixed with p3SR2 15 (an *A. nidulans* amdS gene carrying plasmid) (Tove Christensen et al. Bio/Technology, pp 1419-1422 vol.6, Dec. 1988). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM CaCl<sub>2</sub>, and 10 mM Tris-HCl, pH 20 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, Biochem. Biophys. Acta 113 25 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the 30 second reisolation is stored as a defined transformant.

Test of *A. oryzae* transformants

Each of the *A. oryzae* transformants are inoculated in 10 ml of YPM (cf. below) and propagated. After 2-5 days of incubation 35 at 30°C, the supernatant is removed.

The phytase activity is identified by applying 20 µl supernatant to 4 mm diameter holes punched out in 1% LSB-

agarose plates containing 0.1M Sodiumacetate pH 4.5 and 0.1% Inositol hexaphosphoric acid. The plates are left over night at 37°C. A buffer consisting of 0.1M CaCl<sub>2</sub> and 0.2M Sodium acetate pH 4.5 is poured over the plates and the plates are 5 left at room temperature for 1h. Phytase activity is then identified as a clear zone.

Fed batch fermentation:

Fed batch fermentation was performed in a medium comprising 10 maltodextrin as a carbon source, urea as a nitrogen source and yeast extract. The fed batch fermentation was performed by inoculating a shake flask culture of *A. oryzae* host cells in question into a medium comprising 3.5% of the carbon source and 0.5% of the nitrogen source. After 24 hours of cultivation at 15 pH 7.0 and 34°C the continuous supply of additional carbon and nitrogen sources were initiated. The carbon source was kept as the limiting factor and it was secured that oxygen was present in excess. The fed batch cultivation was continued for 4 days.

20 Isolation of the DNA sequence shown in SEQ ID No. 23:

The phytase encoding part of the DNA sequence shown in SEQ ID No. 23 coding for the phytase of the invention can be obtained from the deposited organism *Escherichia coli* DSM 11312 by extraction of plasmid DNA by methods known in the art 25 (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

Cloning and expression was done by using the Expression cloning in yeast technique as described above.

30

mRNA was isolated from *Peniophora lycii*, CBS No. 686.96, grown as described above.

Mycelia were harvested after 15 days' growth, immediately 35 frozen in liquid nitrogen and stored at -80°C. A library from *Peniophora lycii*, CBS No. 686.96, consisting of approx. 9x10<sup>5</sup> individual clones was constructed in *E. coli* as described with a vector background of 1%. Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-

400 yeast colonies were obtained from each pool.

- Phytase-positive colonies were identified and isolated as described above and inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm. DNA was isolated according to WO 94/14953 and dissolved in 50 µl water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard procedures, and the DNA sequence of the cDNA encoding the phytase was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) and synthetic oligonucleotide primers using an Applied Biosystems ABI PRISM™ 377 DNA Sequencer according to the manufacturers instructions.
- The DNA sequence of the cDNA encoding the phytase is shown in SEQ ID No. 23 and the corresponding amino acid sequence is shown in SEQ ID No. 24. In SEQ ID No. 23 DNA nucleotides from No 1 to No. 1320 define a phytase encoding region.
- The part of the DNA sequence in SEQ ID NO 23, which is encoding the mature part of the phytase is position 91 to 1320, which corresponds to amino acid position 31-439 in SEQ ID NO 24.
- The cDNA is obtainable from the plasmid in DSM 11312.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the phytase in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the phytase gene was purified. The gene was subsequently ligated to pHG414, digested with appropriate restriction enzymes, resulting in the plasmid pA2phy2.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

Test of A. oryzae transformants

Each of the transformants were tested for enzyme activity as described above. Some of the transformants had phytase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of the phytase in *Aspergillus oryzae*.

**EXAMPLE 2**

**Cloning and expression of a phytase from Agrocybe pediades CBS No. 900.96**

Deposited organisms:

*Agrocybe pediades* CBS No. 900.96 comprises a phytase encoding DNA sequence of the invention.

15 *Escherichia coli* DSM NO 11313 contains the plasmid comprising the full length cDNA sequence, coding for a phytase of the invention, in the shuttle vector pYES 2.0.

Isolation of the DNA sequence shown in SEQ ID No. 21:

20 The phytase encoding part of the DNA sequence shown in SEQ ID No. 21 coding for a phytase of the invention can be obtained from the deposited organism *Escherichia coli* DSM 11313 by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

Cloning and expression was done by using the Expression cloning in yeast technique as described in Example 1.

30 mRNA was isolated from *Agrocybe pediades*, CBS No. 900.96, grown as described above with agitation to ensure sufficient aeration.

35 Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library from *Agrocybe pediades*, CBS No. 900.96, consisting of approx. 9x10<sup>5</sup> individual clones was constructed in *E. coli* as described with a vector background of 1%. Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-

400 yeast colonies were obtained from each pool.

Phytase-positive colonies were identified and isolated as described above. cDNA inserts were amplified directly from the 5 yeast colonies and characterized as described in the Materials and Methods section above. The DNA sequence of the cDNA encoding the phytase is shown in SEQ ID No. 21 and the corresponding amino acid sequence is shown in SEQ ID No. 22. In SEQ ID No. 21 DNA nucleotides from No 1 to No. 1362 define 10 the phytase encoding region.

The part of the DNA sequence in SEQ ID NO 21, which is encoding the mature part of the phytase is position 79 to 1362, which correspond to amino acid position 27-453 in SEQ ID 15 NO 22.

The cDNA is obtainable from the plasmid in DSM 11313.

Total DNA was isolated from a yeast colony and plasmid DNA was 20 rescued by transformation of *E. coli* as described above. In order to express the phytase in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the phytase gene was purified. The gene was subsequently ligated 25 to pH414, digested with appropriate restriction enzymes, resulting in the plasmid pA3phy3.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described in Example 1.

30

#### Test of *A. oryzae* transformants

Each of the transformants were tested for enzyme activity as described in Example 1. Some of the transformants had phytase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of 35 the phytase in *Aspergillus oryzae*.

**Example 3**

Cloning and expression of phytases from *Paxillus involutus* CBS 100231 and *Trametes pubescens* CBS 100232

Deposited organisms:

5

*Paxillus involutus* CBS No. 100231 comprises two phytase encoding DNA sequences of the invention and *Trametes pubescens* CBS 100232 comprises a phytase of the invention.

- 10 *Escherichia coli* DSM Nos. 11842, 11843 and 11844 contain the plasmids comprising the full length cDNA sequences, coding for these phytases of the invention, in the shuttle vector pYES 2.0, viz. PhyA1, PhyA2 of *Paxillus involutus* and the phytase of *Trametes pubescens*, respectively.

15

Isolation of the DNA sequences shown in SEQ ID Nos. 25, 27 and 29:

The phytase encoding part of the DNA sequences shown in SEQ ID Nos. 25, 27 and 29 coding for phytases of the invention can be 20 obtained from the deposited organisms *Escherichia coli* DSM 11842, 11843 and 11844, respectively, by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

25

Cloning and expression was done by using the Expression cloning in yeast technique as described in Example 1.

mRNA was isolated from the respective microorganisms, grown 30 under phytase producing conditions, e.g. as described above with agitation to ensure sufficient aeration.

Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. Libraries, 35 consisting of approx.  $9 \times 10^5$  individual clones was constructed in *E. coli* as described with a vector background of 1%. Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

Phytase-positive colonies were identified and isolated as described above. cDNA inserts were amplified directly from the yeast colonies and characterized as described in the Materials and Methods section above. The DNA sequences of the cDNA encoding the phytases are shown in SEQ ID Nos. 25, 27 and 29 and the corresponding amino acid sequences are shown in SEQ ID Nos. 26, 28 and 30, respectively.

10 The cDNA is obtainable from the plasmids in DSM 11842, 11843 and 11844.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In 15 order to express the phytases in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the phytase gene was purified. The gene was subsequently ligated to pHd414, digested with appropriate restriction enzymes.

20

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described in Example 1.

Test of *A. oryzae* transformants

25 Each of the transformants were tested for enzyme activity as described in Example 1. Some of the transformants had phytase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of the phytases in *Aspergillus oryzae*.

30

The two phytases of *Paxillus involutus* CBS 100231 (PhyA1*P.i.* and PhyA2*P.i.*) and the phytase of *Trametes pubescens* CBS 100232 (PhyAT*p.*) have the following characteristics:

	Number of amino acids	Calculated molecular weight (MW)	Isoelectric point (pI)
<b>PhyA1 P.i.</b>	423	46 K	6.4
<b>PhyA2 P.i.</b>	423	45 K	4.5
<b>PhyA T.p.</b>	426	46 K	4.3

**Example 4**

5 **Expression cloning and characterization of five phytase (*phyA*) cDNAs from four basidiomycetes *Agrocybe pediades*, *Peniophora lycii*, *Paxillus involvtus* and *Trametes pubescens*.**

10 Directional cDNA libraries are constructed as described in the previous examples from phytase induced mycelia from the four basidiomycetes *A. pediades*, *P. lycii*, *P. involvtus* and *T. pubescens*, in the yeast expression vector pYES2.0.

15 The cDNA libraries are screened for phytase activity, resulting in isolation of five different phytase cDNAs, *phyA P. lycii*, *phyA A. pediades*, *phyA1 P. involvtus*, *phyA2 P. involvtus*, and *phyA T. pubescens*.

20 Characterization of the *phyA* cDNA from these clones reveals conserved regions apparently specific to the basidiomycete phytases. This indicates that the basidiomycete phytases belong to their own subfamily within the group of fungal phytases.

25 The five new phytases are transformed and overexpressed in *A. oryzae* in order to facilitate the purification and characterization of the recombinant enzymes.

**Isolation of *phyA* cDNAs by expression cloning in yeast.**

30 The fungal strains *A. pediades*, *P. lycii*, *P. involvtus* and *T. pubescens* are cultivated stationary on FG-4 medium (30 g/l soy meal, 15 g/l malto dextrine, 5 g/l bacto peptone, 0.2 g/l pluronic).

The accumulation of total phytase activity in the culture supernatants is monitored on a plate assay as described in the section "Test of *A. oryzae* transformants" of Example 1.

5

Highest levels of phytase activity are detected after five to fifteen days of growth, and therefore poly(A)+RNAs isolated from mycelia harvested according to this, are used to construct four cDNA libraries in the yeast expression vector 10 pYES2.0. Aliquots of the libraries are transformed into *S. cerevisiae* W3124 and the transformants are plated on SC agar containing 2% glucose and incubated at 30°C.

Isolation of poly(A)+ RNA and construction of cDNA libraries 15 is performed as described in Example 1 (the section "Fermentation procedure of *Peniophora lycii* CBS no. 686.96 for mRNA isolation" to the section "Transformation of *Aspergillus oryzae* or *Aspergillus niger*."

20 Identification of positive colonies

Positive colonies are identified as described in Example 1 under the same heading.

Between 20000 and 30000 yeast clones from each library are 25 screened for phytase activity and one to four phytase positive yeast clones are found in each library. The positive colonies correspond to five different phytase genes, *phyA* *P. lycii* (pC1phy2), *phyA* *A. pediades* (pC1phy3), *phyA1* *P. involutus* (pC1phy5), *phyA2* *P. involutus* (pC1phy7), and *phyA* *T. pubescens* 30 (pC1phy6).

Characterization of the *phyA* cDNAs:

The primary structure of the *phyA* cDNA encoding PhyA of *Peniophora lycii* is shown in Fig. 1. The 1593 bp cDNA from 35 pC1phy2 contains a 1320 bp open reading frame (ORF), coding for a 439 residue polypeptide with a calculated molecular weight of 47560. The *phyA* cDNA encodes an 30 amino acid signal peptide. The mature protein has a calculated molecular weight of 44473 and an isoelectric point of pI 4.15. The cDNA and

amino acid sequences are included in the sequence listing, (SEQ ID NO: 23) and (SEQ ID NO: 24), respectively.

The *phyA* cDNA sequence and the deduced sequence of PHYA from 5 *A. pediades* are presented in Fig. 2. The 1501 bp cDNA from pC1phy3 contains a 1362 ORF coding for a 453 residue polypeptide with a 31 amino acid long signal peptide. The 422 amino acid mature protein has a calculated molecular weight of 46781 and an isoelectric point of pI 4.82. The cDNA and amino 10 acid sequences are included in the sequence listing as (SEQ ID NO: 21) and (SEQ ID NO: 22), respectively.

The nucleotide sequences of the *phyA1* and the *phyA2* cDNA cloned from *Paxillus involutus*, and the deduced sequences of 15 PHY1 and PHY2, are shown in Fig. 3. and Fig. 4. respectively.

The 1522 bp insert in pC1phy5 (*phyA1*) contains a 1329 bp ORF coding for a 442 amino acid polypeptide. According to the SignalP V1.1 prediction (Henrik Nielsen, Jacob Engelbrecht, 20 Stren Brunak and Gunnar von Heijne: "Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites," Protein Engineering 10, 1-6 (1997)), the signal peptide consists of 19 amino acids. The mature protein therefore has a predicted molecular weight of 45932 25 and a pI of 6.39. The cDNA and amino acid sequences are included in the sequence listing as (SEQ ID NO: 25) and (SEQ ID NO: 26), respectively.

The plasmid pC1phy7 (*phyA2*) contains a 1642 bp insert with a 30 1329 bp ORF coding for a 442 residue polypeptide. The SignalP V1.1 program referred to above predicts a putative signal peptidase cleavage site between Ala-19 and Ala-20 in the *phyA2* encoded preprotein and thus the predicted molecular weight of the mature protein is 45466 and the predicted isoelectric 35 point is 4.50. The cDNA and amino acid sequences are included in the sequence listing as (SEQ ID NO: 27) and (SEQ ID NO: 28), respectively.

In Fig. 5. the *phyA* cDNA sequence and the deduced sequence of PHYA from *T. pubescens* are shown. The 1536 bp insert in PC1phy6 contains a 1332 bp ORF coding for a 443 residue polypeptide. According to the SignalP V1.1 prediction referred 5 to above, the signal peptide consists of 17 residues. The mature protein therefore consists of 426 amino acids and has a predicted molecular weight of 45905 and a pI of 4.34. The cDNA and amino acid sequences are included in the sequence listing as (SEQ ID NO: 29) and (SEQ ID NO: 30), respectively.

10

#### Conserved basidiomycete phytase regions

The overall identities between known phytases of the phylum Ascomycota and phytases of the invention of the phylum 15 Basidiomycota and are shown in table 1 below ("X/Y" meaning "DNA/peptide" identity as determined by GAP GCGv8).

In this table, the first five phytases in the leftmost column are basidiomycete phytases, whereas the rest are ascomycete 20 phytases.

**Table 1**

Homology of ascomycete and basidiomycete phytases (complete cDNA compared)

	phyA1 P. involvens	phyA2 P. involvens	phyA T. pubescens	phyA A.pediades	phyA P.lycii	phyA A.fumigatus	phyA A.niger	phyA A.terreus	phyA T.lanuginosa	phyA M.thermophila	phyA T. thermophilus	phyA E. nidulans
phyA1 P. involvens	81/85	66/62	58/58	56/56	50/40	51/40	52/41	49/40	54/41	48/37	49/40	
phyA2 P. involvens	81/85	66/63	59/58	57/53	49/39	50/38	51/39	48/39	54/40	50/36	49/39	
phyA T. pubescens	66/62	66/63	60/61	58/51	51/40	50/41	53/39	49/40	53/41	50/39	50/39	
phyA A.pediades	58/58	59/58	60/61	56/52	48/37	49/38	48/38	47/37	50/39	48/37	50/42	
phyA P.lycii	56/56	57/53	58/51	56/52	50/41	51/42	53/41	50/39	54/45	50/43	50/40	
phyA A.fumigatus	50/40	49/39	51/40	48/37	50/41	56/65	64/59	59/51	58/51	62/61	65/67	
phyA A.niger	51/40	50/38	50/41	49/38	51/42	65/65	64/61	58/52	58/48	61/61	64/63	
phyA A.terreus	52/41	51/39	53/39	48/38	53/41	64/59	64/61	56/49	62/47	62/57	63/57	
phyA T.lanuginosa	49/40	48/39	49/40	47/37	50/39	59/52	58/53	56/49	55/46	62/59	59/54	
phyA M.thermophila	54/41	54/40	53/41	50/39	54/45	58/51	58/48	62/47	55/46	57/48	57/51	
PhyA T. thermophilus	48/37	50/36	50/39	48/37	50/43	62/61	61/61	62/57	62/59	57/48	60/59	
phyA E. nidulans	49/40	49/39	50/39	50/42	50/40	65/67	64/63	63/57	59/54	57/51	60/59	

In this experiment, the complete cDNA sequences were compared. According to table 1, the DNA-homology for phytases within the 5 basidiomycetes group is in the range of from 81% to 56% identity, and within the ascomycetes group in the range of from about 65% to 55% identity. Accordingly, the internal group homology seems higher within the group of basidiomycetes phytases as compared to ascomycetes phytases.

10

The DNA homology of the basidiomycet phytases versus the ascomycet phytases, however, is only in the range of from about 54% to 48%. Accordingly, these two groups as such are more different from each other than the difference observed 15 within each group (and this points towards the discrimination between ascomycete phytases and basidiomycete phytases being legitimate).

This relationship is also visualized in the alignments in Fig. 20 6. and Fig. 7.

For some of the phytases of Table 1, Table 2 below shows the results when comparing cDNA sequences of ORF and peptide

sequences of the mature protein (signal peptide cleaved off).

**Table 2**

Homology of selected ascomycete and basidiomycete phytases  
5 (ORF cDNA and mature polypeptide compared)

%ID pep.		phyA1 P. involutus	phyA2 P. involutus	phyA T. pubescens	phyA A. pediades	phyA P. lycii	phyA A. fumigatus	phyA A. niger	phyA A. terreus	phyA T. lanuginosa	phyA M. thermophila
%ID DNA		phyA1 P. involutus	phyA2 P. involutus	phyA T. pubescens	phyA A. pediades	phyA P. lycii	phyA A. fumigatus	phyA A. niger	phyA A. terreus	phyA T. lanuginosa	phyA M. thermophila
phyA1 P. involutus							40	41	41	40	41
phyA2 P. involutus							39	39	39	40	41
phyA T. pubescens							41	42	39	40	42
phyA A. pediades							39	39	41	37	40
phyA P. lycii							42	42	43	41	46
phyA A. fumigatus	50	49	51	48	50						
phyA A. niger	51	50	50	49	51						
phyA A. terreus	52	51	53	48	53						
phyA T. lanuginosa	49	48	49	47	50						
phyA M. thermophila	54	54	53	50	54						

In this table, peptide homologies are indicated in the upper right half of the table, whereas DNA homologies are indicated in the lower left half (both % identity according to GAP GCGv8).  
10

From the alignments at figs. 6 and 7 it is apparent that several sequence motifs are conserved within the five basidiomycete phytases. Based on this alignment several conserved partial sequences have been derived (SEQ ID Nos: 1-14). Still further, some regions of deletions, which are also conserved in the basidiomycete phytases, have also been 15 derived (see e.g claim 5).  
20

Some examples of particularly highly conserved sequences are the so-called Consensus Sequences I, II and III below, the corresponding alignments of which are shown in Tables 3 and 4 25 below. In these tables, identical residues in at least nine of the sequences are indicated by a grey box and identical

residues for the phytases from basidiomycetes are indicated by a white box.

Consensus Sequence I: I-Q-R-H-G-A-R-[F/W]-P-T-S-G-A-X-X-R  
 5 (SEQ ID NO: 3)

Consensus Sequence II: N-W-T-[A/E]-G-F-X-X-A-S  
 (SEQ ID NO: 5)

10 Consensus Sequence III:

F-V-E-S-Q-X-[Y/F]-A-R-X-X-G-X-G-D-F-[E/A]-K-C  
 (SEQ ID NO: 9)

**Table 3**

15 Partial alignments corresponding to consensus sequences I and II

	AA pos.68 - 83 in P.lycii	AA pos.162 - 171 in P.lycii
phyA1 P. involutus	I C R E C A E H F E D S G A T T R	N W T A C G F A S A - - - - S
phyA2 P. involutus	I C R E C A E H F E D S G A A T R	N W T A C G F A S A - - - - S
phyA T. pubescens	I Q R E C A E H F E D S G A A K R	N W T A C G F A L A - - - - S
phyA A. pediades	I Q R E C A E H F E D S G A G T R	N W T E C G F S A A - - - - S
phyA P. lycii	I Q R E C A E H W E D S G A R S R	N W T A C G F G D A - - - - S
phyA A. furnigatus	L S P E C A E H Y D D S S K S K K	K F I E C G Q Q A K L A D P G A -
phyA A. niger	L S P E C A E H Y D D S K G K K	K F I E C G Q S T K L K D P R A C
phyA A. terreus	L A P E C A E H S D H S K T K A	K F V E C G Q T R Q D D H H A N
phyA T. lanuginosa	L S P E C A E H Y D A H K S E V	F F N R E C G Q D A K D R D P R S N
phyA M. thermophila	L S P E C A E H A L K R A A S	N F T Q C G H S A L L A D R G S T

**Table 4**

Partial alignments corresponding to consensus sequence III

5

AA pos.415 - 433 in P.lycii									
phyA1 P. involutus	V	E	S	Q	T	F	A	S	D
phyA2 P. involutus	P	E	S	Q	A	Y	A	S	G
phyA T. pubescens	P	E	S	Q	A	Y	A	N	D
phyA A. pediades	P	E	S	Q	K	Y	A	E	D
phyA P. lycii	P	E	S	Q	T	Y	A	E	D
phyA A. fumigatus	P	K	G	L	S	W	A	S	G
phyA A. niger	R	G	L	S	F	A	S	G	-
phyA A. terreus	A	G	L	S	F	A	Q	A	G
phyA T. lanuginosa	W	I	K	G	L	T	F	Q	G
phyA M. thermophila	I	E	S	M	A	F	R	G	N

10

15

Table 5 below also shows some of the consensus sequences, viz. (SEQ ID NO: 2), (SEQ ID NO: 5) and (SEQ ID NO: 9), respectively, in an alignment as in Fig. 7.

Table 5

Basidiomycete phytase consensus sequences in alignment

AA pos. P. lycii

64

70

75

80

phyA1 P. involutus

N I

R H G A R F

P T

phyA2 P. involutus

N I

R H G A R F

P T

phyA T. pubescens

H I

R H G A R F

P T

phyA A. pediades

N I

R H G A R F

P T

phyA P. lycii

N L

R H G A R W

P T

phyA A. fumigatus

L V

Q V L

S R H G A R Y

P T S S K

phyA A. niger

F A

Q V L

S R H G A R Y

P T D S K

phyA A. terreus

F V

Q V L

A R H G A R S

P T H S K

phyA T. lanuginosa

F V

Q V L

S R H G A R Y

P T A H K

phyA M. thermophila

F A

Q V L

S R H G A R A

P T L K R

AA pos. P. lycii

162

170

171

phyA1 P. involutus

A

G F

A

-

-

phyA2 P. involutus

A

G F

A

-

-

phyA T. pubescens

A

G F

A

-

-

phyA A. pediades

E

G F

S

-

-

phyA P. lycii

T

A G F

G

-

-

phyA A. fumigatus

K

F

D

P G A

-

phyA A. niger

K

F

Q

-

-

phyA A. terreus

K

F

Q

-

-

phyA T. lanuginosa

F

N

T

D P R S N

-

phyA M. thermophila

N

F

S

-

-

AA pos. P. lycii

415

423

431

phyA1 P. involutus

F

V

T

A

E

phyA2 P. involutus

F

V

F

G

C

phyA T. pubescens

F

V

Y

G

C

phyA A. pediades

F

V

Y

E

C

phyA P. lycii

F

V

Y

E

C

phyA A. fumigatus

F

V

Y

G

C

phyA A. niger

F

V

Y

G

C

phyA A. terreus

F

V

Y

G

C

phyA T. lanuginosa

W

I

Y

H

C

phyA M. thermophila

F

I

Y

C

C

Consensus Sequence I (SEQ ID NO: 3), residue position 68 to 83 with the numbering for PHYA *P. lycii* in Fig. 7., is around the active site, and all five basidiomycetes phytases have this 5 consensus sequence I-Q-R-H-G-A-R-[F/W]-P-T-S-G-A-X-X-R with thirteen conserved residues. Still further, four of the five phytases have a fourteenth common residue F75. This is in contrast to the ascomycetes phytases which only have eight conserved residues in the same region (Table 3).

10

When Consensus Sequence II (SEQ ID NO: 5), AA position 162 - 171 in *P. lycii*, is compared to the ascomycete phytases it can be seen that the basidiomycete phytases lack six to seven residues between *P. lycii* F167 (*A. niger* F177) to *P. lycii* 15 P177 (*A. niger* P194) (See Fig. 7) and that the basidiomycetes phytases overall have a much larger degree of conservation with seven identical residues out of ten (Table 3). The ascomycetes phytases have only three conserved residues out of seventeen in the same region.

20

Consensus Sequence III (SEQ ID NO: 9), AA pos. 415-433 in *P. lycii*, consists of nineteen residues with thirteen residues conserved in the basidiomycetes phytases. There are three residues in the consensus sequence that are conserved through 25 all the fungal phytases. In the *P. lycii* sequence the residues are A422, G428, and C433 and for *A. niger* they are A454, G458, and C463. All the basidiomycete phytases have five residues between the conserved alanine and glycine while all the ascomycete phytases only have three (Table 4).

30

#### Expression of PHYA in *Aspergillus oryzae*

In order to obtain high level expression of the PHYA phytases in *Aspergillus oryzae* for further purification and characterization of the protein, the five *phyA* cDNAs from *A. pediades*, *P. lycii*, *P. involutus*, and *T. pubescens* were 35 subcloned into pHD414, a fungal expression vector. The *phyA* cDNA is here inserted 3' to the TAKA-amylase promoter sequence and 5' to the polyA and terminator sequence from the *A. niger* glucoamylase gene. The pHD414 *phyA* constructs were

transformed into *A. oryzae* by co-transformation with the *amdS* selection plasmid (see the section "Transformation of *Aspergillus oryzae* or *Aspergillus niger*" in Example 1). The transformants were screened for phytase activity in the 5 supernatants, and the highest yielding transformants were selected for fermentation.

### Conclusion

The high degree of conserved regions within this group of 10 basidiomycete phytases indicate that they belong to their own subfamily within the group of fungal phytases.

Based on these regions PCR-primers specific for molecular screening of related phytases can be designed (Example 5).

15

### **Example 5**

#### **Molecular screening (primerset 522/538)**

The following degenerate oligonucleotide primers coding for highly conserved regions within the five basidiomycete 20 phytases have been designed for molecular screening:

522 sense primer:

5' - CCC AAG CTT AAY TGG ACN GMN GGN TT - 3'  
(SEQ ID NO: 15)

25

corresponds to amino acids N-W-T-[A,E,D]-G-[F,L] with a CCC and HindIII site 5' tail;

537 sense primer:

30 5' - CCC AAG CTT GAY AAR TWY GGN AC - 3'  
(SEQ ID NO: 16)

corresponds to amino acids D-K-[F,Y]-Y-G-T with a CCC and HindIII site 5' tail;

35

538 anti-sense primer:

5' - GCT CTA GAC RTA RWA YTT RTC NAR RTC - 3'  
(SEQ ID NO: 17)

40 corresponds to amino acids D-[F,L]-D-K-[F,Y]-Y-G with a GC and XbaI site 5' tail;

525 anti-sense primer:

5' - GCT CTA GAC AYT TNK CRA ART CNC C - 3'  
(SEQ ID NO: 18)

5

corresponds to amino acids G-D-F-[A,D,E]-K with a GC and XbaI site 5' tail;

539 sense primer:

10 5' - CCC AAG CTT CAR GTN MAY MTN ATH CA - 3'  
(SEQ ID NO: 19)

corresponds to amino acids Q-V-[N,H]-[I,L,M]-I-[Q,H] with a CCC and HindIII site 5' tail (SEQ ID NO: 15);

15

540 anti-sense primer:

5' - GCT CTA GAC RAA NCC NKC NGT CCA RTT - 3'  
(SEQ ID NO: 20)

20 corresponds to amino acids N-W-T-[A,D,E]-G-F with a GC and XbaI site 5' tail;

wherein N = A, C, G or T; R = A or G; Y = C or T; M = A or C; W = A or T.

25

The design of the primers is based on the alignment in Fig. 7.

For a general reference to the PCR reaction, reference can be had to e.g. Sambrook et al, Molecular Cloning, a Laboratory 30 Manual, 2<sup>nd</sup> edition; or Lubert Stryer: Biochemistry, 4<sup>th</sup> edition, Freeman and Company, New York, 1995, e.g. pp. 132-134.

First, the 522/538 primerset is tested on genomic DNA from 35 selected ascomycetes and basidiomycetes shown in Table 6 below.

The genomic DNA is isolated according to the following procedure:

40

Procedure for isolation of fungal genomic DNA.

1. Grind mycelia in liquid N<sub>2</sub> in a morter
2. Transfer mycelia to an 2.0 ml Eppendorf tube up to the 0.5 ml mark
- 5 3. Add 1.0 ml lysis buffer and mix
4. Add 10 µl 4 mg/ml DNase free RNase A (New England Biolabs)
5. Incubate for 30 min. at 37°C
6. Add 40 µl 16 mg/ml Protease K (New England Biolabs)
- 10 7. Incubate for 1 h. at 50°C with gently shaking
8. Centrifuge for 15 minutes full speed in a microcentrifuge
9. Apply supernatant to a QIAprep-spin column. Spin for 1 min. and discard filtrate
10. Wash with 0.5 ml buffer PB, spin for 1 min. and discard filtrate
- 15 11. Wash with 0.75 ml buffer PE, spin for 1 min. and discard filtrate
12. Drain any existing PE buffer with a quick spin and let dry completely
- 20 13. Place spin column in a clean microfuge tube and elute by adding 125 µl H<sub>2</sub>O. Let sit for 5 min. and then spin for 3 min

## Lysis buffer:

- 25 100 mM EDTA
- 10 mM Tris pH. 8
- 1% Triton X-100
- 200 mM NaCl
- 500 mM Guanidine-HCl

30

For further information on QIAprep spin column, PB buffer and PE buffer please refer to the QIAprep™ Plasmid Handbook from QIAGEN GmbH.

35 Experimental procedure

Approximately 100 to 200 ng genomic DNA or 10-20 ng doublestranded cDNA is used as template for PCR amplification in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 200 µM of each dNTP, 3.5 mM MgCl<sub>2</sub>, 2.5 Units AmpliTaq Gold™,

and 100 pmol of each of the degenerate primers 522 and 538. The total volume is 50 µl. The PCR reaction is carried out in a Perkin - Elmer GeneAmp PCR System 2400. The PCR reaction is performed using a cycle profile of:

5

94 °C - 10 min; 1 cycle

94 °C - 1 min, 60°C - 1 min, 72°C - 30 sec; 2 cycles

94 °C - 1 min, 59°C - 1 min, 72°C - 30 sec; 2 cycles

94 °C - 1 min, 58°C - 1 min, 72°C - 30 sec; 2 cycles

10

-

-

-

94 °C - 1 min, 52°C - 1 min, 72°C - 30 sec; 2 cycles

94 °C - 1 min, 50°C - 1 min, 72°C - 30 sec; 14 cycles

15 72°C - 7 min; 1 cycles

5 µl aliquots of the amplification products are analyzed by electrophoresis in 1.5% agarose gels.

20 Table 6 below shows the results of the test of this primerset, viz. whether a specific PCR band was detected or not.

**Table 6**

Test of primerset 522/538 on genomic DNA from asco- and  
25 basidiomycetes

Microorganism	Phyllum	Strain collection number	PCR band detected
Cladophilus sp.	Ascomycota	CBS 427.97	No
Cytospora sp.	Ascomycota	CBS 424.97	No
Cytospora sp.	Ascomycota	CBS 425.97	No
Gelasinospora sp.	Ascomycota	NN 040455	No
Agrocybe pediades	Basidiomycota	CBS 900.96	Yes
Amylostereum Chailletii	Basidiomycota	NN strain collection	Yes

<i>Bjerkandera adusta</i>	Basidiomycota	CBS 580.95	Yes
<i>Bjerkandera sp.</i>	Basidiomycota	NN strain collection	Yes
<i>Bolbitius aleuritus</i>	Basidiomycota	do	Yes
<i>Cerrena unicolor</i>	Basidiomycota	do	Yes
<i>Coniophora arida</i>	Basidiomycota	do	Yes
<i>Conocybe sp.</i>	Basidiomycota	do	Yes
<i>Coprinus cinereus</i>	Basidiomycota	IFO 30116	Yes
<i>Cystoderma carcharias</i>	Basidiomycota	NN strain collection	Yes
<i>Daedalea quercina</i>	Basidiomycota	NN005877	Yes
<i>Exidia glandulosa</i>	Basidiomycota	CBS 277.96	Yes
<i>Femsjonia sp.</i>	Basidiomycota	NN strain collection	Yes
<i>Fomes fomentarius</i>	Basidiomycota	CBS 276.96	Yes
<i>Hygrophoropsis pallida</i>	Basidiomycota	NN strain collection	Yes
<i>Hypoderma argillaceum</i>	Basidiomycota	do	Yes
<i>Hyphodontia pallidula</i>	Basidiomycota	do	Yes
<i>Hypholoma fasciculare</i>	Basidiomycota	do	Yes
<i>Irpex lacteus</i>	Basidiomycota	do	Yes
<i>Laetisaria arvalis</i>	Basidiomycota	do	Yes
<i>Lyophyllum sp.</i>	Basidiomycota	do	Yes
<i>Marasmiellus ramealis</i>	Basidiomycota	do	Yes
<i>Merismodes sp.</i>	Basidiomycota	do	Yes
<i>Merulius tremellosus</i>	Basidiomycota	do	Yes
<i>Oxyporus corticola</i>	Basidiomycota	do	Yes
<i>Oxyporus sp.</i>	Basidiomycota	CBS 422.97	Yes

<i>Panaeolus semiovatus</i>	Basidiomycota	CBS 819.95	Yes
<i>Paxillus involutus</i>	Basidiomycota	CBS 100231	Yes
<i>Peniophora cinerea</i>	Basidiomycota	NN007373	Yes
<i>Peniophora lycii</i>	Basidiomycota	CBS 686.96	Yes
<i>Peniophora quercina</i>	Basidiomycota	NN 009335	Yes
<i>Podaxis pistillaris</i>	Basidiomycota	ATCC 38868	Yes
<i>Scizophyllum commune</i>	Basidiomycota	NN strain collection	Yes
<i>Scizophyllum</i> sp.	Basidiomycota	CBS 443.97	Yes
<i>Skeletocutis</i> sp.	Basidiomycota	NN strain collection	Yes
<i>Steccherinum ochraceum</i>	Basidiomycota	do	Yes
<i>Stereum subtomentosum</i>	Basidiomycota	do	Yes
<i>Strobilurus tenacellus</i>	Basidiomycota	do	Yes
<i>Stropharia cubensis</i>	Basidiomycota	ATCC 13966	Yes
<i>Trametes hirsuta</i>	Basidiomycota	DSM 2987	Yes
<i>Trametes pubescens</i>	Basidiomycota	CBS 100232	Yes
<i>Trametes zonatella</i>	Basidiomycota	NN strain collection	Yes
<i>Trechispora farinaceae</i>	Basidiomycota	do	Yes
<i>Trichaptum fuscoviolaceum</i>	Basidiomycota	do	Yes
<i>Typhula setipes</i>	Basidiomycota	do	Yes
<i>Volvariella speciosa</i>	Basidiomycota	do	Yes

**Example 6****Molecular screening (other primersets)**

Primersets 522/525, 539/540, 539/538, 539/525 and 537/525 are tested as described in Example 5 above, using 100 pmol of each 5 of the sense and anti-sense degenerate primers. Touchdown PCR is used for amplification (ref: R.H.Don et al. (1991), Nucleic Acid Research, Vol. 19, No. 14) modified for the AmpliTaq Gold (TM). The PCR reaction is performed using a cycle profile of:

10 94 C - 10 min; 1 cycle

94 C - 1 min, 60C - 1 min, 72C - 1.5 min; 2 cycles

94 C - 1 min, 59C - 1 min, 72C - 1.5 min; 2 cycles

94 C - 1 min, 58C - 1 min, 72C - 1.5 min; 2 cycles

-

15 -

-

94 C - 1 min, 52C - 1 min, 72C - 1.5 min; 2 cycles

94 C - 1 min, 50C - 1 min, 72C - 1.5 min; 14 cycles

72C - 7 min; 1 cycle.

20

**Example 7****Purification and sequencing of PCR bands**

The PCR fragments can be purified and sequenced using the Jet sorb Gel extraction Kit (Genomed GmbH, Germany) according to

25 the manufacturer's instructions. The nucleotide sequences of the amplified PCR fragments are determined directly on the purified PCR products using 200-300 ng as template, the Taq deoxy-terminal cycle sequencing kit (Perkin-Elmer, USA), fluorescent labeled terminators and 5 pmol sequence primer on 30 a ABI PRISM<sup>TM</sup> 377 DNA Sequencer, Perkin Elmer.

The PCR fragments generated with primer set 522/538, and with approximately 10-20 ng of doublestranded cDNA from *Schizophyllum* sp. CBS 443.97 as template, was purified and 35 sequenced as described above and the DNA sequence was deduced to (5'- to 3'-):

TCTGCCGCATCTGACGGTGTCTATAACCCCGTCCTAACCTGATTATATCAGAAGAGCTTAA  
CGACACCCTCGATGATGCGATGTGCCCGAACGTCGGCGAACGGACGCCAACGGACGAAT

GGACGTCTATTCAGCAGGCCATCGCTGAGCGTCTGAACAAACAACGCCGTGGGCCTAAC  
CTGACCACCACGAACGTTACAACCTCATGTCTTATGCCCTTCGACACGCTTGCAGAGGA  
GACGCCGAGCCCCTCTGCGATCTTT (SEQ ID NO: 31)

5 and translated into amino acid sequence:

SAASDGVYNPVNLIISEELNDLDDAMCPNVGESDAQTDEWTSIYAAPIAERLNNNAVGAN  
LTNTNVYNLMSLCPFDTLAKETPSPFCDLF (SEQ ID NO: 32).

10 The doublestranded cDNA was synthesized as described in Example 1.

**Example 8**

Purification and characterization of the phytase from  
15 *Peniophora lycii* expressed in *Aspergillus oryzae*

The *Peniophora lycii* phytase was expressed in and excreted from *Aspergillus oryzae* IFO 4177.

Filter aid was added to the culture broth which was filtered  
20 through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear solution. The filtrate was concentrated by ultrafiltration on 3kDa cut-off polyethersulphone membranes followed by diafiltration with distilled water to reduce the conductivity.  
25 The pH of the concentrated enzyme was adjusted to pH 7.5. The conductivity of the concentrated enzyme was 1.2 mS/cm.

The phytase was applied to a Q-sepharose FF column equilibrated in 20mM Tris/CH<sub>3</sub>COOH, pH 7.5 and the enzyme was  
30 eluted with an increasing linear NaCl gradient (0 → 0.5M). The phytase activity eluted as a single peak. This peak was pooled and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 1.5M final concentration. A Phenyl Toyopearl 650S column was equilibrated in 1.5M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10mM succinic acid/NaOH, pH 6.0 and the phytase was  
35 applied to this column and eluted with a decreasing linear (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient (1.5 → 0M). Phytase containing fractions were pooled and the buffer was exchanged for 20mM Tris/CH<sub>3</sub>COOH, pH 7.5 on a Sephadex G25 column. The G25 filtrate was applied

to a Q-sepharose FF column equilibrated in 20mM Tris/CH<sub>3</sub>COOH, pH 7.5. After washing the column extensively with the equilibration buffer, the phytase was eluted with an increasing linear NaCl gradient (0 → 0.5M). The phytase activity was pooled and the buffer was exchanged for 20mM Tris/CH<sub>3</sub>COOH, pH 7.5 by dialysis. The dialysed phytase was applied to a SOURCE 30Q column equilibrated in 20mM Tris/CH<sub>3</sub>COOH, pH 7.5. After washing the column thoroughly with the equilibration buffer a phytase was eluted with an increasing linear NaCl gradient (0 → 0.3M). Fractions from the SOURCE 30Q column were analyzed by SDS-PAGE and pure phytase fractions were pooled.

The *Peniophora* phytase migrates in the gel as a band with M<sub>r</sub> = 15 67 kDa. N-terminal amino acid sequencing of the 67 kDa component was carried out following SDS-PAGE and electroblotting onto a PVDF-membrane. The following N-terminal amino acid sequence could be deduced:

20 Leu-Pro-Ile-Pro-Ala-Gln-Asn-

The sequence corresponds to amino acid residues 31-37 in the cDNA derived amino acid sequence.

25 Accordingly a mature amino acid sequence of the phytase when expressed in *Aspergillus* is supposed to be no. 31-439 of SEQ ID no 24.

#### **Example 9**

30 **Further characterization of the purified phytase of *Peniophora lycii***

The phytase of *Peniophora lycii* was expressed in *Aspergillus* and purified as described in Example 8.

35

The phytase activity is measured using the following assay: 10 µl diluted enzyme samples (diluted in 0.1 M sodium acetate, 0.01 % Tween20, pH 5.5) were added into 250 µl 5 mM sodium

phytate (Sigma) in 0.1 M sodium acetate, 0.01 % Tween20, pH 5.5 (pH adjusted after dissolving the sodium phytate; the substrate was preheated) and incubated for 30 minutes at 37°C. The reaction was stopped by adding 250 µl 10 % TCA and free phosphate was measured by adding 500 µl 7.3 g FeSO<sub>4</sub> in 100 ml molybdate reagent (2.5 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O in 8 ml H<sub>2</sub>SO<sub>4</sub> diluted to 250 ml). The absorbance at 750 nm was measured on 200 µl samples in 96 well microtiter plates. Substrate and enzyme blanks were included. A phosphate standard curve was also included (0-2 mM phosphate). 1 FYT equals the amount of enzyme that releases 1 µmol phosphate/min at the given conditions.

Temperature profiles were obtained by running the assay at various temperatures (preheating the substrate).

15

Temperature stability was investigated by preincubating the phytases in 0.1 M sodium phosphate, pH 5.5 at various temperatures before measuring the residual activity.

20 The pH-stability was measured by incubating the enzyme at pH 3 (25 mM glycine-HCl), pH 4-5 (25 mM sodium acetate), pH 6 (25 mM MES), pH 7-9 (25 mM Tris-HCl) for 1 hour at 40 °C, before measuring the residual activity.

25 The pH-profiles were obtained by running the assay at the various pH using the same buffer-systems (50 mM, pH was re-adjusted when dissolving the substrate).

The results of the above pH-profile, pH-stability, 30 temperature-profile and temperature stability studies are shown in figs. 8, 9, 10 and 11, respectively. From fig. 9 it appears that the phytase of *Peniophora lycii* is very stable (i.e. more than 80% of the maximum activity retained) for 1 hour at 40°C in the whole range of pH 3-9. And as regards the 35 temperature stability results shown at fig. 11, it appears that at 60-80°C some 50-60% of the residual activity still remains. This fact is contemplated to be due to the enzyme being surprisingly capable of refolding following its thermal denaturation. The degree of refolding will depend on the exact

conditions (pH, enzyme concentration).

Fig. 12 shows the result of differential scanning calorimetry (DSC) measurements on the *Peniophora* phytase. In DSC the heat consumed to keep a constant temperature increase in the sample-cell is measured relative to a reference cell. A constant heating rate is kept (e.g. 90°C/hour). An endothermal process (heat consuming process - e.g. the unfolding of an enzyme/protein) is observed as an increase in the heat transferred to the cell in order to keep the constant temperature increase. DSC was performed using the MC2-apparatus from MicroCal. Cells were equilibrated 20 minutes at 20°C before scanning to 90°C at a scan rate of 90°/h. Samples of around 2.5 mg/ml *Peniophora* phytase in 0.1 M sodium acetate, pH 5.5 were loaded.

#### Example 10

##### Determination of the specific activity of the *Peniophora* phytase

- 20 The specific activity is determined on a highly purified sample of the phytase (the purity was checked beforehand on an SDS poly acryl amide gel showing the presence of only one component).
- 25 The protein concentration in the phytase sample was determined by amino acid analysis as follows: An aliquot of the phytase sample was hydrolyzed in 6N HCl, 0.1% phenol for 16 h at 110 C in an evacuated glass tube. The resulting amino acids were quantified using an Applied Biosystems 420A amino acid analysis system operated according to the manufacturers instructions. From the amounts of the amino acids the total mass - and thus also the concentration - of protein in the hydrolyzed aliquot can be calculated.
- 35 The activity is determined in the units of FYT. One FYT equals the amount of enzyme that liberates 1 micromol inorganic phosphate from phytate (5 mM phytate) per minute at pH 5.5, 37 C; assay described e.g. in example 11.

The specific activity is calculated to 987 FYT/mg enzyme protein.

5 Example 11

**Time-resolved product-profiling of phytase-catalyzed hydrolysis of phytic acid by  $^1\text{H}$  NMR spectroscopy**

The hydrolysis of phytic acid (PA) catalyzed by the Peniophora phytase and by a commercial Aspergillus niger phytase (Phytase 10 Novo®) was investigated (27 mM phytate, 1 FYT/ml, pH 5.5 and 3.5, and 27°C) by  $^1\text{H}$  NMR profiling the product mixture in the course of 24 hours.

In the following  $\text{Ins}(p,q,r,\dots)\text{P}_n$  denotes myo-inositol carrying 15 in total n phosphate groups attached to positions p, q, r,.. For convenience  $\text{Ins}(1,2,3,4,5,6)\text{P}_6$  (phytic acid) is abbreviated PA. Please refer, however, to the section "Nomenclature and position specificity of phytases" in the general part of this application.

20

The technique provide specific information about initial points of attack by the enzyme on the PA molecule, as well as information about the identity of the end product. On the other side the evolving patterns of peaks reflecting the 25 composition of the intermediate product mixtures, provide a qualitative measure, a finger print, suitable for identification of similarities and differences between individual enzymes.

30 NMR, like most other analytical methods, can distinguish between stereo-isomers which are not mirror images (diastereomers), but not between a set of isomers, which are mirror-images (enantiomers), since they exhibit identical NMR spectra.

35

Thus,  $\text{Ins}(1,2,4,5,6)\text{P}_5$  (3-phosphate removed) exhibits a NMR spectrum different from  $\text{Ins}(1,2,3,4,5)\text{P}_5$  (6-phosphate removed) because the isomers are diastereomers.

However, the NMR spectra of Ins(1,2,4,5,6)P<sub>5</sub> and Ins(2,3,4,5,6)P<sub>5</sub> (1-phosphate removed) are identical because the isomers are enantiomers. The same holds for the pair 5 Ins(1,2,3,4,5)P<sub>5</sub> and Ins(1,2,3,5,6)P<sub>5</sub> (4-phosphate removed).

Thus, by NMR it is not possible to distinguish between a 3- and a 1-phytase, and it is not possible to distinguish between a 6- and a 4-phytase (or a L-6- and a D-6-phytase using the 10 lowest-locant rule).

Biased by the description of 3- and 6-phytases in the literature, we have used the terms 3- and 6-phytases for our enzymes, but, though unlikely, we do not actually know if we 15 have a 1- and a 4-phytase instead.

#### Experimental.

NMR spectra were recorded at 300 K (27°C) on a Bruker DRX400 instrument equipped with a 5 mm selective inverse probe head. 20 16 scans preceded by 4 dummy scans were accumulated using a sweep width of 2003 Hz (5 ppm) covered by 8 K data points. Attenuation of the residual HOD resonance was achieved by a 3 seconds presaturation period. The spectra were referenced to the HOD signal ( $\delta$  4.70).

25

PA samples for NMR analysis were prepared as follows: PA (100 mg, Phytic acid dipotassium salt, Sigma P-5681) was dissolved in deionized water (4.0 ml) and pH adjusted to 5.5 or 3.5 by addition of aqueous NaOH (4 N). Deionized water was added (ad 30 5 ml) and 1 ml portions, each corresponding to 20 mg of phytic acid, were transferred to screw-cap vials and the solvent evaporated (vacuum centrifuge). The dry samples were dissolved in deuterium oxide (2 ml, Merck 99.5% D) and again evaporated to dryness (stored at -18°C until use).

35

For NMR analysis one 20 mg phytic acid sample was dissolved in deuterium oxide (1.0 ml, Merck 99.95% D). The solution was transferred to a NMR tube and the <sup>1</sup>H NMR spectrum recorded.

Enzyme solution (1 FTU, dissolved in/diluted, as appropriate, with deuterium oxide) was added followed by thorough mixing (1 minute).  $^1\text{H}$  NMR spectra were recorded immediately after addition of enzyme ( $t=0$ ), then after 5, 10, 15, 20, 25, 30, 5 45, 60, 75, 90, 105, 120, 135 150, 165, 180, 195, 210 minutes (= 3.5 hours), 4.5, 5.5 6.5, 7.5, 8.5, 9.5, 11.5, 13.5, 15.5, 17.5, 19.5, 21.5, and 23.5 hours. The pH in the NMR tube was measured. Additional spectra were acquired after 48 and 120 hours (5 days), where a portion of substrate (PA, 6 mg) was 10 added to probe if the enzyme retained its catalytic activity.

By means of 2D NMR analysis of inositol phosphate mixtures obtained by partial digestion of PA, in conjunction with published NMR data (Scholz, P.; Bergmann, G., and Mayr, G.W.: 15 *Methods in Inositide Research* (Ed. Irvine, R.F.), pp. 65-82, Raven Press, Ltd., New York (1990)), characteristic  $^1\text{H}$  NMR signals attributable to  $\text{Ins}(1,2,3,4,5,6)\text{P}_6$  (PA),  $\text{Ins}(1,2,4,5,6)\text{P}_5$ ,  $\text{Ins}(1,2,3,4,5)\text{P}_5$ ,  $\text{Ins}(1,2,5,6)\text{P}_4$ ,  $\text{Ins}(1,2,6)\text{P}_3$ ,  $\text{Ins}(1,2)\text{P}_2$ , and  $\text{Ins}(2)\text{P}$ , were identified and 20 permitted relative quantification of these species during the course of the reaction.

Stacked plots of product profiles for the *Aspergillus* phytase and the *Peniophora* phytase covering 24 hours of reaction time 25 at pH 5.5 is presented in Fig. 13 and Fig. 14, respectively.

The signal at  $\delta$  3.25(t) represents H-5 in  $\text{Ins}(1,2)\text{P}_2$  whereas the signal at  $\delta$  3.18(t) represents H-5 in  $\text{Ins}(2)\text{P}$ .  $\text{Ins}(1,2)\text{P}_2$  starts accumulating after about 4 hours of reaction time with 30 the *Aspergillus* phytase and after about 1 hours of reaction time with the *Peniophora* phytase.  $\text{Ins}(2)\text{P}$  is observed after about 10 hours of reaction with the *Aspergillus* phytase and after about 3 hours of reaction with the *Peniophora* phytase. After 24 hours of reaction the amount or level of  $\text{Ins}(1,2)\text{P}_2$  is 35 very low for both phytases, whereas the amount of  $\text{Ins}(2)\text{P}$  is maximum for both phytases after 24 hours.

Accordingly, the profiles observed after 24 hours of reaction

time demonstrate that both phytases degrade PA to Ins(2)P.

For both enzymes the reaction mixture at 24 h comprised in addition to Ins(2)P minor amounts of Ins(1,2)P<sub>2</sub>. Prolonged 5 reaction times (several days) resulted in disappearance of the residual Ins(1,2)P<sub>2</sub>, but the fully dephosphorylated species, inositol (Ins), was not observed at all. The observation is not explained by irreversible inhibition/denaturation of the enzyme, since the enzymes retained their catalytic activities 10 for prolonged periods, as demonstrated by their ability to digest fresh portions of PA added to the NMR tubes after keeping them 5 days at room temperature.

Turning now to figs. 15 and 16, these depict in more detail 15 the profiles evolving at pH 5.5 during the initial 4.5 hours. It is inferred from fig. 10 that H-3 in Ins(1,2,4,5,6)P<sub>5</sub> (designated A) shows a signal at  $\delta$  3.66(dd), H-6 in Ins(1,2,3,4,5)P<sub>5</sub> (B) a signal at  $\delta$  3.87(t) and H-3 in Ins(1,2,5,6)P<sub>4</sub> (C) a signal at  $\delta$  3.56(dd). Now, compound A 20 corresponds to phosphate in position 3 having been hydrolyzed, B position 6 and C position 3 and 4.

It is apparent from fig. 15 that compound A appears as the major primary product ( $t=5$  min) using the Aspergillus phytase, 25 whereas compound B does not appear. Compound C appears after 20-25 minutes.

From fig. 16 (the Peniophora phytase) one infers that compound B appears as the major primary product ( $t=5$  min) using the 30 Peniophora phytase.

The signals at  $\delta$  4.82(dt, H-2), 4.38 (q, H-4/H-6), 4.13(q, H-5) and 4.11(dt, H1/H3) are attributable to the substrate, phytic acid, PA. Comparing figs. 15 and 16 it is apparent, 35 that these peaks diminish faster with the Peniophora phytase than with the Aspergillus phytase.

These differences are highlighted in Fig. 17, which present the profiles observed after 20 min at pH 5.5 with the above indicated diagnostic signals (A,B,C) labelled.

- 5 Fig. 18 shows the final result (under these conditions) of the hydrolysis of phytic acid at pH 5.5 (i.e. corresponding to the upper line of figs. 13 and 14). All signals labelled at the upper Peniophora embodiment represent the compound Ins(2)P, viz. the protons thereof, from the right to the left: H-5, H1  
10 and H3, H4 and H6 and finally H-2. Relative intensity: 1:2:2:1. The corresponding signals are found in the bottom embodiment of Aspergillus. This means that the end product is in both embodiments Ins(2)P. However, a minor amount of Ins(1,2)P<sub>2</sub> is also detected in both embodiments, the  
15 corresponding peaks being indicated at the Aspergillus embodiment only.

Marked differences are observed:

*Aspergillus*: The initial major product was identified as  
20 Ins(1,2,4,5,6)P<sub>5</sub> (A) followed by appearance of Ins(1,2,5,6)P<sub>4</sub>(C), and Ins(1,2,6)P<sub>3</sub> (D) (H-3 at  $\delta$  3.49(dd) after 1½ hours) corresponding to consecutive removal of the phosphate groups in the 3-, 4- and 5-positions. The concentration of Ins(1,2)P<sub>2</sub> (E) builds up slowly starting at 4  
25 hours and decreases very steeply between 12 and 14 hours with a concomitant rapid increase of the Ins(2)P (F) level. This is visualized in Fig. 11 representing the time dependent concentration of Ins(1,2)P<sub>2</sub> and Ins(2)P, respectively, determined by measuring the area under the signals  
30 corresponding to H-5 in Ins(1,2)P<sub>2</sub> ( $\delta$  3.25(t)) and Ins(2)P. ( $\delta$  3.18 (t)), respectively, relative to the area under the signals corresponding to the substrates (t=0).

*Peniophora*: At pH 5.5 only the 6-position is initially  
35 attacked. A characteristic feature is that PA is digested at a faster rate compared to the Aspergillus phytase. Additional characteristic features are that the end product, Ins(2)P (F) appears very early (3 hours) and builds up slowly, in contrast

to the very steep increase in the Ins(2)P-level towards the end of the reaction observed for the Aspergillus phytase.

Fig. 19 is a plot similar to fig. 17, but at pH 3.5.  
5 Surprisingly, at this pH the Peniophora phytase turns up to have high initial affinity to the 6- as well as the 3-position of PA (B as well as A are observed), probably with a slight preference for the 6-position.

10 The data generated permit i.a. the following conclusions:

At pH 5.5 as well as 3.5 the Aspergillus phytase attacks with a high degree of selectivity PA in the 3-position, whereas the Peniophora phytase at pH 5.5 with a high degree of selectivity  
15 attacks PA in the 6-position, at pH 3.5 however it seems to hydrolyze the phosphate groups at the 3- and 6-positions at comparable rates.

At pH 5.5, the Peniophora phytase digests PA at a faster rate  
20 compared to the Aspergillus phytase.

The end-product is, at pH 3.5 as well as 5.5, under the conditions applied, Ins(2)P (F).

25 The overall reaction rates ( $PA \rightarrow Ins(2)P$ ) were comparable, approximately 20 hours (Fig. 20; pH 5.5).

Accordingly, the Aspergillus phytase prove to be an essentially clean 3-phytase, whereas the Peniophora phytase at  
30 pH 5.5 appear to be an essentially clean 6-phytase and at pH 3.5 a phytase of a hitherto unknown type, viz a 3+6-phytase.

The exact configuration of myo-inositol tetrakisphosphate produced by partial hydrolysis af phytic acid with the  
35 Peniophora phytase could be determined as outlined below, also allowing us to conclude whether the Peniophora phytase is a D-, L- or a D/L-6-phytase.

Other ways of determining the exact specificity is by determining optical rotation or using a chiral HPLC column.

1. HPLC-isolation of myo-inositol tetrakisphosphate produced by partial degradation of phytic acid with the Peniophora phytase. Desalting (ion exchange, dialysis, (2), (4) and (9) and references herein)
2. NMR analysis to check purity (i), determine whether several diastereomer tetrakisphosphates are produced (ii), and determine which of these are produced (iii)
3. Synthesis of relevant polyols using reduction by boronhydrid (BH) of the corresponding carbonhydrates (10)
4. Disintegration using periodate, reduction by boronhydrid and dephosphorylation following (2). Identification of polyol using HPLC
5. Oxidation of polyol using L-iditol dehydrogenase and final identification of carbonhydrid using HPLC.

#### References:

- (2) Van der Kaay et al, Biochem. J., 312 (1995), 907-910
- (4) Irving et al, J. Bacteriology, 112 (1972), 434-438
- (9) Stevens, L.R. in "Methods in Inositide Research" (Irvine, R.F. Ed.), 9-30 (1990), Raven Press, Ltd., New York.
- (10) Stephens, L. et al, Biochem. J., 249 (1988), 271-282

25

#### Example 12

##### Comparative assay, *Aspergillus* and *Peniophora* phytase Release of inorganic phosphate from corn

- The present example gives a simple assay for the phytase catalyzed liberation of phosphorous from corn at pH 3.5 and 5.5. Two parameters have been focused on - velocity and level of P-liberation.

35 Materials and methods:

Corn was obtained from North Carolina State University (sample No. R27), and ground at a mill (Bühler Universal) at point 6.8.

A corn-suspension (16.7% w/w) was prepared by weighing 20 g of ground corn into a 250 ml blue cap bottle and adding 100 ml of buffer.

5 The following buffer was used:

pH 5.5: 0.22 M acetate-buffer

The pH value of 3.5 was adjusted by 8N HCl/NaOH.

10 Enzymes tested: Two phytases was tested: A commercial phytase of *Aspergillus niger* (Phytase Novo®) and a *Peniophora* phytase of the invention, purified as described in example 2.

Dosage: All enzymes were applied at 25 FYT/20 g corn (correspond to 1250 FYT/kg).

15

The bottles with the corn suspension were closed by caps, and immediately placed in a water bath at 37°C and subjected to constant stirring. pH was measured at this stage and again after 24 hours. After 30 min of stirring a sample of 5 ml was 20 collected.

Then the phytase enzymes were added at a dosage of 25 FYT/20 g of corn.

25 Samples were then collected 5, 10, 15, 20, 25, 30, 40, 50, 60 and 120 min after the addition of the phytases, and the content of released P determined as follows:

Phytase containing samples were diluted 1+4 in buffer. Then the 30 samples were centrifuged at 3000 rpm for 5 min, and 1.0 ml of the supernatant was collected. 2.0 ml buffer and 2.0 ml MoV stop solution (cfr. the FYT assay of Example 6) was added. The samples were placed in a refrigerator at 3-5°C until all samples could be measured at the spectrophotometer at 415nm.

35

pH was measured at time 0 and 20 hours.

For the determinations a phosphate standard or stock solution of

50mM was used prepared. 0.5, 1.0, 1.5 and 2.0 ml stock solution is diluted to a total volume of 50 ml using buffer. 3.0 ml of each solution is added 2.0 ml MoV stop solution.

5 Two experiments were conducted: at pH 5.5 and at pH 3.5. The analysis results are shown at figs. 21 and 22 (pH 5.5 and 3.5, respectively). At these figures, symbol "◆" represents the control experiment, "▲" the Peniophora phytase and "■" the Aspergillus phytase.

10

Results and discussion:

Fig. 21 (pH 5.5) shows, that at this pH the Peniophora phytase liberates P from corn at significantly improved rate as compared to the Aspergillus phytase.

15

From fig. 22 (pH 3.5) it is clearly apparent that at this pH the Peniophora phytase is much faster in the liberation of phosphorous from ground corn as compared to the Aspergillus phytase (0-120 minutes).

20

The passage time of the digestive system of for instance chickens / broilers is normally is of the order of magnitude of 30 minutes to 2 hours, so the observed difference is for sure important, whatever the pH. Nevertheless the pH value of 3.5 is more 25 relevant in this respect than the pH 5.5 value.

This implies that the Peniophora enzyme is surprisingly more efficient than the known Aspergillus phytase as a P-liberator in the digestive system of e.g. broilers.

30

**Example 13**

**Fermentation, purification and characterization of the phytase of Agrocybe pediades expressed in yeast**

A seed culture is prepared by incubation of the yeast strain 35 in 100 ml medium A at 250 rpm over night at 30°C. 100 ml medium B is inoculated with 2 ml seed culture and the strains incubate for 7 to 12 days at 30°C 250 rpm.

*Agrocybe pediades* phytase was expressed in yeast as described in Example 2. The yeast clone comprises a cloned sequence encoding a phytase of the invention having the amino acid 5 sequence shown in SEQ ID No 22.

Filter aid was added to the culture supernatant which was filtered through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a 10 clear solution. The filtrate was concentrated by ultrafiltration on 3kDa cut-off polyethersulphone membranes and refiltered on a germ filter plate. The pH of the filtrate was adjusted to pH 7.5 and the conductivity was adjusted to 2mS/cm by dilution with distilled water.

15

The phytase was applied to a Q-sepharose FF column equilibrated with 20mM Tris/CH<sub>3</sub>COOH, pH 7.5 and the enzyme was eluted with an increasing linear NaCl gradient (0 → 0.5M). The phytase containing fractions from the Q-sepharose column 20 were pooled and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 1.3M final concentration. A Phenyl Toyopearl 650S column was equilibrated with 1.3M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10mM succinic acid/NaOH, pH 6.0 and the phytase was applied to this column and eluted with a decreasing linear (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient (1.3 → 0M). Phytase 25 containing fractions were pooled and buffer was exchanged with 20mM Tris/CH<sub>3</sub>COOH, pH 7.5 on a Sephadex G25 column. Phytase was further purified on a SOURCE Q column equilibrated with 20mM Tris/CH<sub>3</sub>COOH, pH 7.5, and eluted with a linear NaCl gradient (0 → 0.5M). Finally, phytase containing fractions from the 30 SOURCE Q column were pooled, concentrated on a 10kDa cut-off regenerated cellulose membrane, and applied to a Superdex 200 column equilibrated in 25mM CH<sub>3</sub>COOH/NaOH, 100mM NaCl, pH 5.0.

Fractions from the Superdex 200 column were analyzed by SDS-PAGE. The phytase migrates in the gel as a very broad and diffuse band with approx. Mr = 150 kDa indicating that the 35 enzyme was highly glycosylated.

N-terminal amino acid sequencing of the 150 kDa component was carried out following SDS-PAGE and electroblotting onto a PVDF membrane.

5

Two N-terminal sequences could be deduced in the relative amounts of approximately 4:1 (upper sequence:lower sequence) :

Val-Gln-Pro-Phe-Phe-Pro-Pro-Gln-Ile-Gln-Asp-Ser-Trp-Ala-Ala-  
10 Tyr-Thr-Pro-Tyr-Tyr-Pro-Val-Gln-

and

Thr-Phe-Val-Gln-Pro-Phe-Phe-Pro-Pro-Gln-Ile-Gln-Asp-Ser-Trp-  
15 Ala-Ala-Tyr-Thr-Pro-Tyr-Tyr-Pro-

The two N-terminal amino acids "Val" and "Thr" are found in position 27 and 25, respectively, in SEQ ID NO 22. This indicates that the mature phytase enzyme of the invention, when 20 expressed in yeast, starts at position 27 or 25 in SEQ ID No 22.

Accordingly the mature amino acid sequence of the phytase when expressed in yeast is supposed to be no. 27-453 or 25-453 of 25 SEQ ID no 22.

#### EXAMPLE 14

##### Purification and characterization of the phytase from *Agrocybe pediades* expressed in *Aspergillus oryzae*

30 The *Agrocybe pediades* phytase was expressed in and excreted from *Aspergillus oryzae* IFO 4177.

Filter aid was added to the culture broth which was filtered through a filtration cloth. This solution was further filtered 35 through a Seitz depth filter plate resulting in a clear solution. The filtrate was concentrated by ultrafiltration on 3kDa cut-off polyethersulphone membranes followed by diafiltration with distilled water to reduce the conductivity. The pH of the concentrated enzyme was adjusted to pH 7.5.

The phytase was applied to a Q-sepharose FF column equilibrated in 20mM Tris/CH<sub>3</sub>COOH, pH 7.5 and the enzyme was eluted with an increasing linear NaCl gradient (0 → 0.5M).

5 The phytase activity eluted as a single peak. This peak was pooled and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 1.3M final concentration. A Phenyl Toyopearl 650S column was equilibrated in 1.3M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10mM succinic acid/NaOH, pH 6.0 and the phytase was applied to this column and eluted with a decreasing linear

10 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient (1.3 → 0M). Phytase containing fractions were pooled and the buffer was exchanged for 20mM Tris/CH<sub>3</sub>COOH, pH 7.5 by dialysis. The phytase was applied to a SOURCE 30Q column equilibrated in 20mM Tris/CH<sub>3</sub>COOH, pH 7.5 and the enzyme was eluted with an increasing linear NaCl gradient (0 →

15 0.25M). The phytase activity was pooled and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 1.6M final concentration. A SOURCE Phenyl column was equilibrated in 1.6M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 25mM succinic acid/NaOH, pH 6.0 and the phytase was applied to this column and eluted with a decreasing linear (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient (1.6 → 0M). Fractions from

20 the SOURCE Phenyl column were analyzed by SDS-PAGE and pure phytase fractions were pooled. The phytase pool was dialysed against 20mM Tris/CH<sub>3</sub>COOH, pH 7.5, applied to a HighTrap Q column equilibrated in the same buffer, and stepeluted with 20mM Tris/CH<sub>3</sub>COOH, 0.5M NaCl, pH 7.5.

25

The Agrocybe phytase migrates on SDS-PAGE as a band with Mr = 60 kDa.

N-terminal amino acid sequencing of the 60 kDa component was

30 carried out following SDS-PAGE and electroblotting onto a PVDF-membrane. Two N-terminal amino acid sequences could be deduced in relative amounts of approximately 2:1 (upper sequence:lower sequence).

35 Phe-Pro-Pro-Gln-Ile-Gln-Asp-Ser-Trp-Ala-Ala-Tyr-Thr-Pro-Tyr-Tyr-Pro-Val-Gln-

and

Gln-Pro-Phe-Phe-Pro-Pro-Gln-Ile-Gln-Asp-Ser-Trp-Ala-Ala-Tyr-Thr-Pro-Tyr-Tyr-

5

The upper sequence corresponds to amino acid residues 31-49 in the cDNA derived amino acid sequence while the lower sequence corresponds to amino acid residues 28-46.

10 Accordingly the mature amino acid sequence of the phytase when expressed in *Aspergillus* is supposed to be no. 31-453 or 28-453 of SEQ ID no 22.

Accordingly, summing up the results of example 13 and the  
15 present example, in SEQ ID NO 21, the following sequences are  
phytase encoding sub-sequences: position 79 to 1362, 73-1362,  
91-1362 or 82-1362 (i.e. corresponding to amino acid positions  
27-453, 25-453, 31-453 or 28-453, respectively, in SEQ ID NO  
22).

20

Accordingly, there is a slight variability in the N-terminal sequence of the mature phytase enzyme. This variability is observed as well when the enzyme is expressed in a single strain, as when expressed in different strains. In yeast, the  
25 mature phytase enzyme starts at amino acid no. 27 or 25 (relative abundance about 80%:20%, respectively); in *Aspergillus* the mature phytase enzyme starts at amino acid no. 31 or 28 (relative abundance: about 65%:35%, respectively).

30 Example 15

**Characterization of the purified phytase of Agrocybe pediades**  
The phytase of *Agrocybe pediades* was expressed in *Aspergillus* and purified as described in Example 14.

35 The phytase activity is measured using the following assay:

10 µl diluted enzyme samples (diluted in 0.1 M sodium acetate, 0.01 % Tween20, pH 5.5) were added into 250 µl 5 mM sodium phytate (Sigma) in 0.1 M sodium acetate, 0.01 % Tween20, pH 5.5 (pH adjusted after dissolving the sodium phytate; the

substrate was preheated) and incubated for 30 minutes at 37 °C. The reaction was stopped by adding 250 µl 10 % TCA and free phosphate was measured by adding 500 µl 7.3 g FeSO<sub>4</sub> in 100 ml molybdate reagent (2.5 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O in 8 ml H<sub>2</sub>SO<sub>4</sub> diluted to 250 ml). The absorbance at 750 nm was measured on 200 µl samples in 96 well microtiter plates. Substrate and enzyme blanks were included. A phosphate standard curve was also included (0-2 mM phosphate). 1 FYT equals the amount of enzyme that releases 1 µmol phosphate/min at the given conditions.

Temperature profiles were obtained by running the assay at various temperatures (preheating the substrate).

Temperature stability was investigated by preincubating the phytases in 0.1 M sodium phosphate, pH 5.5 at various temperatures before measuring the residual activity.

The pH-stability was measured by incubating the enzyme at pH 3 (25 mM glycine-HCl), pH 4-5 (25 mM sodium acetate), pH 6 (25 mM MES), pH 7-9 (25 mM Tris-HCl) for 1 hour at 40 °C, before measuring the residual activity.

The pH-profiles were obtained by running the assay at the various pH using the same buffer-systems (50 mM, pH was re-adjusted when dissolving the substrate).

The results of the above pH-profile, pH-stability, temperature-profile and temperature stability studies are shown in fig. 23, 24, 25 and 26, respectively.

From fig. 23 it appears that the phytase of Agrocybe pediades has a reasonable activity at pH 3-6 (i.e. more than 50% of the maximum activity). At pH 4-6 more than 70% of the maximum activity is found, at pH 5-6 more than 90%. Optimum pH seems to be in the area of pH 5.5-6.

It is apparent from fig. 24 that the phytase of Agrocybe pediades is very stable (i.e. more than 80% of the maximum

activity retained) for 1 hour at 40°C in the whole range of pH 3-9.

As regards the temperature profile, it is apparent from 5 fig. 25, that the Agrocybe pediades phytase has a reasonable activity at temperatures of 35-55°C (i.e. more than 60% of the maximum activity), whereas at temperatures of 40-52°C the activity is more than 70% of the maximum activity, and the optimum temperature is close to 50°C.

10

And finally, as regards the temperature stability results shown at fig. 26, the phytase is very stable at temperatures of 0 to about 55°C (i.e. more than 60% residual activity). A sharp decline in residual activity is seen after preincubation 15 at 60°C. Anyhow, at 60°C at least 20%, preferably 25% and more preferably 30% of the residual activity still remains. Also at pre-incubation temperature above 60°C, e.g. at 70°C and 80°C, a surprisingly high residual activity remains, viz. more than 20%, preferably more than 30%, especially more than 40% 20 remains.

This fact is contemplated to be due to the enzyme being surprisingly capable of refolding following its thermal denaturation. The degree of refolding will depend on the exact 25 conditions (pH, enzyme concentration).

Fig. 27 shows the result of differential scanning calorimetry (DSC) measurements on the Agrocybe phytase.

30 In DSC the heat consumed to keep a constant temperature increase in the sample-cell is measured relative to a reference cell. A constant heating rate is kept (e.g. 90°C/hour). An endothermal process (heat consuming process - e.g. the unfolding of an enzyme/protein) is observed as an 35 increase in the heat transferred to the cell in order to keep the constant temperature increase.

DSC was performed using the MC2-apparatus from MicroCal. Cells were equilibrated 20 minutes at 20°C before scanning to 90°C

at a scan rate of 90°/h. Samples of around 2.5 mg/ml Agrocybe phytase in 0.1 M sodium acetate, pH 5.5 were loaded.

The temperature stability studies were confirmed by DSC, since from fig. 5 it is apparent that the Agrocybe phytase has a denaturation or "melting" temperature of about 58°C at pH 5.5. The re-scan of the Agrocybe phytase shows a minor peak at 58°C, and this is also indicative of the fact that a fraction of the enzyme is actually refolded folding the thermal inactivation in the first scan.

#### Example 16

#### Time-resolved product-profiling of phytase-catalyzed hydrolysis of phytic acid by $^1\text{H}$ NMR spectroscopy

15

The hydrolysis of phytic acid (PA) catalyzed by the Agrocybe phytase and by a commercial Aspergillus niger phytase (Phytase Novo®) was investigated (27 mM phytate, 1 FYT/ml, pH 5.5, and 27°C) by  $^1\text{H}$  NMR profiling the product mixture in the course of 20 24 hours.

In the following ( $\text{Ins}(p,q,r,\dots)\text{P}_n$ ) denotes myo-inositol carrying in total n phosphate groups attached to positions p, q, r,.. For convenience  $\text{Ins}(1,2,3,4,5,6)\text{P}_6$  (phytic acid) is abbreviated 25 PA. Please refer, however, to the section "Nomenclature and position specificity of phytases" in the general part of this application.

The technique provide specific information about initial 30 points of attack by the enzyme on the PA molecule, as well as information about the identity of the end product. On the other side the evolving patterns of peaks reflecting the composition of the intermediate product mixtures, provide a qualitative measure, a finger print, suitable for 35 identification of similarities and differences between individual enzymes.

NMR, like most other analytical methods, can distinguish

between stereo-isomers which are not mirror images (diastereomers), but not between a set of isomers, which are mirror-images (enantiomers), since they exhibit identical NMR spectra.

5

Thus,  $\text{Ins}(1,2,4,5,6)\text{P}_5$  (3-phosphate removed) exhibits a NMR spectrum different from  $\text{Ins}(1,2,3,4,5)\text{P}_5$  (6-phosphate removed) because the isomers are diastereomers.

10 However, the NMR spectra of  $\text{Ins}(1,2,4,5,6)\text{P}_5$  and  $\text{Ins}(2,3,4,5,6)\text{P}_5$  (1-phosphate removed) are identical because the isomers are enantiomers. The same holds for the pair  $\text{Ins}(1,2,3,4,5)\text{P}_5$  and  $\text{Ins}(1,2,3,5,6)\text{P}_5$  (4-phosphate removed).

15 Thus, by NMR it is not possible to distinguish between a 3- and a 1-phytase, and it is not possible to distinguish between a 6- and a 4-phytase (or a L-6- and a D-6-phytase using the lowest-locant rule).

20 Biased by the description of 3- and 6-phytases in the literature, we have used the terms 3- and 6-phytases for our enzymes, but, though unlikely, we do not actually know if we have a 1- and a 4-phytase instead.

25 Experimental.

NMR spectra were recorded at 300 K (27°C) on a Bruker DRX400 instrument equipped with a 5 mm selective inverse probe head. 16 scans preceded by 4 dummy scans were accumulated using a sweep width of 2003 Hz (5 ppm) covered by 8 K data points. 30 Attenuation of the residual HOD resonance was achieved by a 3 seconds presaturation period. The spectra were referenced to the HOD signal ( $\delta$  4.70).

PA samples for NMR analysis were prepared as follows: PA (100 mg, Phytic acid dipotassium salt, Sigma P-5681) was dissolved in deionized water (4.0 ml) and pH adjusted to 5.5 by addition of aqueous NaOH (4 N). Deionized water was added (ad 5 ml) and 1 ml portions, each corresponding to 20 mg of phytic

acid, were transferred to screw-cap vials and the solvent evaporated (vacuum centrifuge). The dry samples were dissolved in deuterium oxide (2 ml, Merck 99.5% D) and again evaporated to dryness (stored at -18°C until use).

5

For NMR analysis one 20 mg phytic acid sample was dissolved in deuterium oxide (1.0 ml, Merck 99.95% D). The solution was transferred to a NMR tube and the <sup>1</sup>H NMR spectrum recorded. Enzyme solution (1 FTU, dissolved in/diluted, as appropriate, 10 with deuterium oxide) was added followed by thorough mixing (1 minute). <sup>1</sup>H NMR spectra were recorded immediately after addition of enzyme (t=0), then after 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, 120, 135 150, 165, 180, 195, 210 minutes (= 3.5 hours), 4.5, 5.5 6.5, 7.5, 8.5, 9.5, 11.5, 13.5, 15.5, 15 17.5, 19.5, 21.5, and 23.5 hours. The pH in the NMR tube was measured. Additional spectra were acquired after 48 and 120 hours (5 days), where a portion of substrate (PA, 6 mg) was added to probe if the enzyme retained its catalytic activity.

20 By means of 2D NMR analysis of inositol phosphate mixtures obtained by partial digestion of PA, in conjunction with published NMR data (Scholz, P.; Bergmann, G., and Mayr, G.W.: *Methods in Inositide Research* (Ed. Irvine, R.F.), pp. 65-82, Raven Press, Ltd., New York (1990)), characteristic <sup>1</sup>H NMR 25 signals attributable to Ins(1,2,3,4,5,6)P<sub>6</sub> (PA), Ins(1,2,4,5,6)P<sub>5</sub>, Ins(1,2,3,4,5)P<sub>5</sub>, Ins(1,2,5,6)P<sub>4</sub>, Ins(1,2,6)P<sub>3</sub>, Ins(1,2)P<sub>2</sub>, and Ins(2)P, were identified and permitted relative quantification of these species during the course of the reaction.

30

Stacked plots of product profiles for the Aspergillus phytase and the Agrocybe phytase covering 24 hours of reaction time is presented in Fig. 28 and Fig. 29, respectively.

35 The signal at δ 3.25(t) represents H-5 in Ins(1,2)P<sub>2</sub> whereas the signal at δ 3.18(t) represents H-5 in Ins(2)P. Ins(1,2)P<sub>2</sub> starts accumulating after about 4 hours of reaction time with the Aspergillus phytase and after about 2 hours of reaction

time with the Agrocybe phytase. Ins(2)P is observed after about 10 hours of reaction with the Aspergillus phytase and after about 5 hours of reaction with the Agrocybe phytase. After 24 hours of reaction the amount or level of Ins(1,2)P<sub>2</sub> is 5 very low for both phytases, whereas the amount of Ins(2)P is maximum for both phytases after 24 hours.

Accordingly, the profiles observed after 24 hours of reaction time demonstrate that both phytases degrade PA to Ins(2)P. The 10 fully dephosphorylated species, inositol (Ins), was not observed at all.

For both enzymes the reaction mixture at 24 h comprised in addition to Ins(2)P minor amounts of Ins(1,2)P<sub>2</sub>. Prolonged 15 reaction times (several days) resulted in disappearance of the residual Ins(1,2)P<sub>2</sub>, but the fully dephosphorylated species, inositol (Ins), was not observed at all. The observation is not explained by irreversible inhibition/denaturation of the enzyme, since the enzymes retained their catalytic activities 20 for prolonged periods, as demonstrated by their ability to digest fresh portions of PA added to the NMR tubes after keeping them 5 days at room temperature.

Turning now to figs. 30 and 31, these depict in more detail 25 the profiles evolving during the initial 4.5 hours. It is inferred from fig. 32 that H-3 in Ins(1,2,4,5,6)P<sub>5</sub> (designated A) shows a signal at δ 3.66(dd), H-6 in Ins(1,2,3,4,5)P<sub>5</sub> (B) a signal at δ 3.87(t) and H-3 in Ins(1,2,5,6)P<sub>4</sub> (C) a signal at δ 3.56(dd). Now, compound A corresponds to phosphate in position 30 3 having been hydrolyzed, B position 6 and C position 3 and 4.

It is apparent from fig. 30 that compound A appears as the major primary product (t=5 min) using the Aspergillus phytase, whereas compound B does not appear. Compound C appears after 35 20-25 minutes.

From fig. 31 (the Agrocybe phytase) one infers that compound A as well as compound B appear very early, i.e. within the first

15 minutes, probably more of the compound B than A.

The signals at  $\delta$  4.82(dt, H-2), 4.38 (q, H-4/H-6), 4.13(q, H-5) and 4.11(dt,H1/H3) are attributable to the substrate, 5 phytic acid, PA. Comparing figs. 30 and 31 it is apparent, that these peaks diminish much faster (i.e. within an hour) with the Agrocybe phytase than with the Aspergillus phytase.

These differences are highlighted in Fig. 32, which present 10 the profiles observed after 20 min with the above indicated diagnostic signals (A,B,C) labelled.

Fig. 33 shows the final result (under these conditions) of the hydrolysis of phytic acid (i.e. corresponding to the upper 15 line of figs. 28 and 29). All signals labelled at the upper Agrocybe embodiment represent the compound Ins(2)P, viz. the protons thereof, from the right to the left: H-5, H1 and H3, H4 and H6 and finally H-2. Relative intensity: 1:2:2:1. The corresponding signals are found in the bottom embodiment of 20 Aspergillus. This means that the end product is in both embodiments Ins(2)P. However, a minor amount of Ins(1,2)P<sub>2</sub> is also detected in both embodiments, the corresponding peaks being indicated at the Aspergillus embodiment only.

25 Marked differences are observed:

Aspergillus: The initial major product was identified as Ins(1,2,4,5,6)P<sub>5</sub> (A) followed by appearance of Ins(1,2,5,6)P<sub>4</sub>(C), and Ins(1,2,6)P<sub>3</sub> (D) (H-3 at  $\delta$  3.49(dd) after 1½ hours) corresponding to consecutive removal of the 30 phosphate groups in the 3-, 4- and 5-positions. The concentration of Ins(1,2)P<sub>2</sub> (E) builds up slowly starting at 2 hours and decreases very steeply between 12 and 14 hours with a concomitant rapid increase of the Ins(2)P (F) level. This is visualized in Fig. 34 and 35, representing the time dependent 35 concentration of Ins(1,2)P<sub>2</sub> and Ins(2)P, respectively, constructed from slices along the time-dimension in Fig. 28-29 at the chemical shift values ( $\delta$ ) of H-5 in Ins(1,2)P<sub>2</sub> and Ins(2)P, respectively (note that the time scale is only linear

in segments).

Agrocybe: Both the 3- and 6-positions are initially attacked, with some preference for the 6-position. A characteristic 5 feature is that PA is digested at a faster rate compared to the Aspergillus phytase. Additional characteristic features are that the end product, Ins(2)P (F) appear very early (5 hours) and builds up slowly, in contrast to the very steep increase in the Ins(2)P-level towards the end of the reaction 10 observed for the Aspergillus phytase.

The data generated permit i.a. the following conclusions:

The Aspergillus phytase attacks with a high degree of 15 selectivity PA in the 3-position, whereas the Agrocybe phytase appear less specific.

The Agrocybe phytase digests PA at a faster rate compared to the Aspergillus phytase.

20

The end-product is in both cases, under the conditions applied, Ins(2)P (F).

The overall reaction rates (PA $\rightarrow$ Ins(2)P) were comparable, 25 approximately 20 hours (Fig. 35).

Accordingly, the Aspergillus phytase prove to be an essentially clean 3-phytase, whereas the Agrocybe phytase appear to be less specific, however, with some preference for 30 the 6-position.

By application of 2D-homo- and heteronuclear ( $^1\text{H}$ ,  $^{13}\text{C}$ ) correlation techniques, the latter circumventing problems with severely overlapping  $^1\text{H}$ -resonances by taking advantage of the 35 larger chemical shift dispersion of the  $^{13}\text{C}$ -nuclei, in combination with suitable computer software, it would in principle be possible to identify and quantify intermediates present at any given time and thereby completely map out the

reaction sequence. In other words, curves like those shown in Fig. 34 and 35 representing concentration as a function of time, could in theory be constructed for other intermediate inositol phosphates.

5

### Example 17

#### Comparative assay, Aspergillus and Agrocybe phytase Release of inorganic phosphate from corn

- 10 The present example gives a simple assay for the phytase catalyzed liberation of phosphorous from corn at pH 3.5 and 5.5. Two parameters have been focused on - velocity and level of P-liberation.

15

#### Materials and methods:

Corn was obtained from North Carolina State University (sample No. R27), and ground at a mill (Bühler Universal) at point 6.8.

- 20 A corn-suspension (16.7% w/w) was prepared by weighing 20 g of ground corn into a 250 ml blue cap bottle and adding 100 ml of buffer.

The following buffers were used:

- 25 pH 5.5: 0.22 M acetate-buffer  
pH 3.5: 0.05 M citrate-buffer.

Enzymes tested: Two phytases was tested: A commercial phytase of Aspergillus niger (Phytase Novo®) and an Agrocybe phytase of the 30 invention, purified as described in example 3 and 4.

Dosage: All enzymes were applied at 25 FYT/20 g corn (correspond to 1250 FYT/kg).

35 The bottles with the corn suspension were closed by caps, and immediately placed in a water bath at 37°C and subjected to constant stirring. pH was measured at this stage and again after 24 hours. After 30 min of stirring a sample of 5 ml was collected.

Then the phytase enzymes were added at a dosage of 25 FYT/20 g of corn.

- 5 Samples were then collected 10, 20, 30, 40, 50, 60, 120 min, and approx. 20 hours after the addition of the phytases, and the content of release P determined as follows:

Phytase containing samples were diluted 1+4 in buffer. Then the  
10 samples were centrifuged at 3000 rpm for 5 min, and 1.0 ml of the supernatant was collected. 2.0 ml buffer and 2.0 ml MoV stop solution (cfr. the FYT assay of Example 15) was added. The samples were placed in a refrigerator at 3-5°C until all samples could be measured at the spectrophotometer at 415nm.

15

pH was measured at time 0 and 20 hours.

For the determinations a phosphate standard or stock solution of 50mM was used prepared. 0.5, 1.0, 1.5 and 2.0 ml stock solution  
20 is diluted to a total volume of 50 ml using buffer. 3.0 ml of each solution is added 2.0 ml MoV stop solution.

Two experiments were conducted: at pH 5.5 and at pH 3.5. The analysis results are shown at figs. 36 and 37 (pH 5.5 and 3.5,  
25 respectively). At these figures, symbol "◆" represents the control experiment, "▲" the Agrocybe phytase and "■" the Aspergillus phytase.

#### Results and discussion:

30 Figs. 36 and 37 clearly show that the Agrocybe phytase initially liberates phosphorous from ground corn faster than the Aspergillus phytase at pH 5.5 and at pH 3.5.

However, after 40 min. at pH 5.5 (fig. 36) and after 120 min. at  
35 pH 3.5 (fig. 37), the Aspergillus phytase is approximately at the same level of released phosphate as is the Agrocybe phytase.

But considering the passage time of the digestive system of for instance chickens / broilers, which normally is of the order of magnitude of 30 minutes to 2 hours, this difference is for sure important. Besides, it should be mentioned, that the pH value of 5 3.5 is more relevant in this respect than the pH 5.5 value.

This implies that the Agrocybe enzyme is surprisingly more efficient than the known Aspergillus phytase as a P-liberator in the digestive system of e.g. broilers.

10

#### Example 18

#### Purification and characterization of the phytases from *Paxillus involutus* and *Trametes pubescens*

15 The PhyA1 phytase from *Paxillus involutus*

The *Paxillus involutus* PhyA1 phytase was expressed in and excreted from *Aspergillus oryzae* IFO 4177 as described in Examples 3 and 1.

20 Filter aid was added to the culture broth which was filtered through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear solution. The filtrate was concentrated by ultrafiltration on 3kDa cut-off polyethersulphone membranes and the phytase was 25 transferred to 10mM succinic acid/NaOH, pH 6.0 on a Sephadex G25 column. The pH of the G25 filtrate was adjusted to pH 7.0.

The phytase was applied to a Q-sepharose FF column equilibrated in 20mM HEPES/NaOH, pH 7.0. The enzyme turned out 30 to be in the run-through from the column.  $(\text{NH}_4)_2\text{SO}_4$  was added to the run-through to 2.0M final concentration. A Butyl Toyopearl 650S column was equilibrated in 2.0M  $(\text{NH}_4)_2\text{SO}_4$ , 10mM CH<sub>3</sub>COOH/NaOH, pH 5.5 and the phytase was applied to this column and eluted with a decreasing linear  $(\text{NH}_4)_2\text{SO}_4$  gradient (2.0 → 35 0M). Phytase containing fractions were pooled and the buffer was exchanged for 20mM HEPES/NaOH, pH 7.5 on a Sephadex G25 column. The G25 filtrate was applied to a Q-sepharose FF column equilibrated in 20mM HEPES/NaOH, pH 7.5. After washing

the column extensively with the equilibration buffer, the phytase was eluted with an increasing linear NaCl gradient (0 → 0.5M). The phytase activity was pooled and the buffer was exchanged for 20mM Tris/CH<sub>3</sub>COOH, pH 8.0 on a Sephadex G25 column. The G25 filtrate was applied to a SOURCE 30Q column equilibrated in 20mM Tris/CH<sub>3</sub>COOH, pH 8.0. After washing the column thoroughly with the equilibration buffer en phytase was eluted with an increasing linear NaCl gradient (0 → 0.3M). Phytase containing fractions were pooled and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 2.0M final concentration. A Phenyl Toyopearl 650S column was equilibrated in 2.0M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10mM CH<sub>3</sub>COOH/NaOH, pH 5.5 and the phytase was applied to this column and eluted with a decreasing linear (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient (2.0 → 0M). Phytase containing fractions were pooled and reapplied to the same Phenyl Toyopearl column after adding (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 2.0M final concentration. Fractions from the second Phenyl Toyopearl column were analyzed by SDS-PAGE and pure phytase fractions were pooled.

The *Paxillus involutus* PhyA1 phytase migrates on SDS-PAGE as a band with M<sub>r</sub> = 65 kDa. N-terminal amino acid sequencing of the 65 kDa component was carried out following SDS-PAGE and electroblotting onto a PVDF-membrane. The following N-terminal amino acid sequence could be deduced.

25

Ser-Val-Pro-Lys-Asn-Thr-Ala-Pro-Thr-Phe-Pro-Ile-Pro

The sequence corresponds to amino acid residues 21-33 in the cDNA derived amino acid sequence.

30

Accordingly a mature amino acid sequence of the phytase when expressed in *Aspergillus* is supposed to be no. 21-442 of SEQ ID no. 26. Accordingly, the predicted signal peptide at Fig. 3 does not correspond with the actual signalpeptide when this phytase is expressed in *Aspergillus*. This is also so for the indications regarding mature peptide of the sequence listing under the headings SEQ ID NOs: 25 and 26.

The PhyA2 phytase from Paxillus involutus

The *Paxillus involutus* PhyA2 phytase was expressed in and excreted from *Aspergillus oryzae* IFO 4177 as described in 5 Examples 3 and 1.

Filter aid was added to the culture broth which was filtered through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear 10 solution. The filtrate was concentrated by ultrafiltration on 3kDa cut-off polyethersulphone membranes and  $(\text{NH}_4)_2\text{SO}_4$  was added to 2.0M final concentration.

The phytase was applied to a Phenyl sepharose FF column 15 equilibrated in 2.0M  $(\text{NH}_4)_2\text{SO}_4$ , 20mM  $\text{CH}_3\text{COOH}/\text{NaOH}$ , pH 5.5 and the enzyme was eluted with a decreasing linear  $(\text{NH}_4)_2\text{SO}_4$  gradient ( $2.0 \rightarrow 0\text{M}$ ). The phytase activity eluted as a single peak. This peak was pooled and  $(\text{NH}_4)_2\text{SO}_4$  was added to 2.0M final concentration. A Butyl Toyopearl 650S column was 20 equilibrated in 2.0M  $(\text{NH}_4)_2\text{SO}_4$ , 10mM  $\text{CH}_3\text{COOH}/\text{NaOH}$ , pH 5.5 and the phytase was applied to this column and eluted with a decreasing linear  $(\text{NH}_4)_2\text{SO}_4$  gradient ( $2.0 \rightarrow 0\text{M}$ ). Phytase containing fractions were pooled and the buffer was exchanged 25 for 20mM HEPES/ $\text{NaOH}$ , pH 7.0 on a Sephadex G25 column. The G25 filtrate was applied to a Q-sepharose FF column equilibrated in 20mM HEPES/ $\text{NaOH}$ , pH 7.0. After washing the column extensively with the equilibration buffer, the phytase was eluted with an increasing linear NaCl gradient ( $0 \rightarrow 0.5\text{M}$ ). The phytase activity was pooled and the buffer was exchanged 30 for 20mM HEPES/ $\text{NaOH}$ , pH 7.0 by dialysis. The dialysed phytase was applied to a SOURCE 30Q column equilibrated in 20mM HEPES/ $\text{NaOH}$ , pH 7.0. After washing the column thoroughly with the equilibration buffer en phytase was eluted with an increasing linear NaCl gradient ( $0 \rightarrow 0.3\text{M}$ ). Fractions from 35 the SOURCE 30Q column were analyzed by SDS-PAGE and pure phytase fractions were pooled.

The *Paxillus involutus* PhyA2 phytase migrates on SDS-PAGE as a band with  $M_r = 52$  kDa. N-terminal amino acid sequencing of the 52 kDa component was carried out following SDS-PAGE and electroblotting onto a PVDF-membrane. The following N-terminal 5 amino acid sequence could be deduced.

Asn-Ile-Ala-Pro-Lys-Phe-

The sequence corresponds to amino acid residues 25-30 in the 10 cDNA derived amino acid sequence.

Accordingly a mature amino acid sequence of the phytase when expressed in *Aspergillus* is supposed to be no. 25-442 of SEQ ID no. 28. Accordingly, the predicted signal peptide at 15 Fig. 4 does not correspond with the actual signalpeptide when this phytase is expressed in *Aspergillus*. This is also so for the indications regarding mature peptide of the sequence listing under the headings SEQ ID NOS: 27 and 28.

20 The phytase from *Trametes pubescens*

The *Trametes pubescens* phytase was expressed in and excreted from *Aspergillus oryzae* IFO 4177.

Filter aid was added to the culture broth which was filtered 25 through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear solution. The filtrate was concentrated by ultrafiltration on 10kDa cut-off polyethersulphone membranes followed by diafiltration with distilled water to reduce the conductivity. 30 The pH of the concentrated enzyme was adjusted to pH 6.0 and the conductivity was adjusted to that of 10mM succinic acid/NaOH, pH 6.0 by dilution with deionised water.

The phytase was applied to a Q-sepharose FF column 35 equilibrated in 10mM succinic acid/NaOH, pH 6.0 and the enzyme was eluted with an increasing linear NaCl gradient (0 → 0.5M). The phytase activity eluted as a single peak. This peak was pooled and  $(\text{NH}_4)_2\text{SO}_4$  was added to 2.0M final concentration.

A Butyl Toyopearl 650S column was equilibrated in 2.0M  $(\text{NH}_4)_2\text{SO}_4$ , 10mM  $\text{CH}_3\text{COOH}/\text{NaOH}$ , pH 5.5 and the phytase was applied to this column and eluted with a decreasing linear  $(\text{NH}_4)_2\text{SO}_4$  gradient ( $2.0 \rightarrow 0\text{M}$ ). Phytase containing fractions were pooled and the buffer was exchanged for 10mM succinic acid/ $\text{NaOH}$ , pH 6.5 on a Sephadex G25 column. The G25 filtrate was applied to a Q-sepharose FF column equilibrated in 10mM succinic acid/ $\text{NaOH}$ , pH 6.5. After washing the column extensively with the equilibration buffer, the phytase was eluted with an increasing linear NaCl gradient ( $0 \rightarrow 0.3\text{M}$ ). The phytase activity was pooled and the buffer was exchanged for 10mM succinic acid/ $\text{NaOH}$ , pH 7.0 on a Sephadex G25 column. The G25 filtrate was applied to a SOURCE 30Q column equilibrated in 10mM succinic acid/ $\text{NaOH}$ , pH 7.0. After washing the column thoroughly with the equilibration buffer en phytase was eluted with an increasing linear NaCl gradient ( $0 \rightarrow 0.2\text{M}$ ). Fractions from the SOURCE 30Q column were analyzed by SDS-PAGE and pure phytase fractions were pooled.

The *Trametes pubescens* phytase migrates on SDS-PAGE as a band with  $M_r = 57$  kDa. N-terminal amino acid sequencing of the 57 kDa component was carried out following SDS-PAGE and electroblotting onto a PVDF-membrane. The following N-terminal amino acid sequence could be deduced.

25

Xxx-Ala-Cys-Leu-Asp-Val-Thr-Arg-Asp-(Ala/Val)-Gln-

The sequence corresponds to amino acid residues 31-41 in the cDNA derived amino acid sequence.

30

Accordingly a mature amino acid sequence of the phytase when expressed in *Aspergillus* is supposed to be no. 31-443 of SEQ ID no. 30. Accordingly, the predicted signal peptide at Fig. 5 does not correspond with the actual signalpeptide when this phytase is expressed in *Aspergillus*. This is also so for the indications regarding mature peptide of the sequence listing under the headings SEQ ID NOS: 29 and 30.

The molecular weights (kDa) of the three phytases of *Trametes pubescens* and PhyA2 and PhyA1 of *Paxillus involutus* are 65, 55 and 65 kDa, respectively.

5

The pH profiles of these three phytases are similar to Figs. 8 and 23 for the *Peniophora* and the *Agrocybe* phytases (optimum pH around 5-6; very little activity at pH above 7). As compared to the known phytase of *Aspergillus ficuum* (in this test having a temperature optimum of around 50°C) PhyA1 of *Paxillus involutus* has a slightly higher temperature optimum around 60°C, while PhyA2 has a temperature optimum of around 40°C. The thermostability of the PhyA1 phytase of *Paxillus involutus* is comparable to that of the *Aspergillus ficuum* phytase, and better than that of the PhyA2 phytase of *Paxillus involutus* and the phytase of *Trametes pubescens*. Following 60 minutes incubation at 70°C and 80°C, respectively, the residual activity of PhyA1 is around 60% and 40%, respectively.

20

In DSC, Td's of around 48°C, 59°C and 55°C are found for the phytases PhyA2 and PhyA1 of *Paxillus involutus* and for the phytase of *Trametes pubescens*, respectively.

25 The specific activities are surprisingly high for all these phytases, viz. 1450, 1370 and 810 FYT/mg enzyme protein for the phytases of *Trametes pubescens*, *Paxillus involutus* PhyA2 and PhyA1, respectively ( $A_{280}$  of 3.58; 5.72 and 3.08; FYT/ml of 5184, 7808 and 2497; assumed extinction coefficient of 1  
30 1/(g x cm), respectively).

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: Novo Nordisk A/S
- (B) STREET: Novo Alle
- (C) CITY: DK-2880 Bagsvaerd
- (D) STATE: Denmark
- (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): 2880
- (G) TELEPHONE: +45 4444 8888
- (H) TELEFAX: +45 4449 3256

## (ii) TITLE OF INVENTION: Phytase polypeptides

## (iii) NUMBER OF SEQUENCES: 32

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (v) FRAGMENT TYPE: internal

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 2
- (D) OTHER INFORMATION:/product= "OTHER"  
/note= "X in position 2 is Y or F"

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 3
- (D) OTHER INFORMATION:/product= "OTHER"  
/note= "X in position 3 is F or Y"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Pro	Xaa	Xaa	Pro	Xaa	Xaa	Xaa	Tyr	Xaa	Xaa	Pro	Pro
1							5				10

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (v) FRAGMENT TYPE: internal

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 3
- (D) OTHER INFORMATION:/note= "X in position 3 is N or H"

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site

(B) LOCATION:4  
(D) OTHER INFORMATION:/note= "X in position 4 is I or L"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site  
(B) LOCATION:12  
(D) OTHER INFORMATION:/note= "X in position 12 is F or W"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gln Val Xaa Xaa Ile Gln Arg His Gly Ala Arg Xaa Pro Thr Ser Gly  
1 5 10 15  
Ala

## (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 16 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

## (ix) FEATURE:

(A) NAME/KEY: Modified-site  
(B) LOCATION:8  
(D) OTHER INFORMATION:/note= "X in position 8 is F or W"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ile Gln Arg His Gly Ala Arg Xaa Pro Thr Ser Gly Ala Xaa Xaa Arg  
1 5 10 15

## (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 13 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

## (ix) FEATURE:

(A) NAME/KEY: Modified-site  
(B) LOCATION:4  
(D) OTHER INFORMATION:/note= "X in position 4 is D or A"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site  
(B) LOCATION:5  
(D) OTHER INFORMATION:/note= "X in position 5 is S or T"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site  
(B) LOCATION:6  
(D) OTHER INFORMATION:/note= "X in position 6 is A or S"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site  
(B) LOCATION:7  
(D) OTHER INFORMATION:/note= "X in position 7 is T or N"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION:11  
(D) OTHER INFORMATION:/note= "X in position 11 is A or E"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Arg Val Val Xaa Xaa Xaa Asn Trp Thr Xaa Gly Phe  
1 5 10

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

(A) NAME/KEY: Modified-site  
(B) LOCATION:4  
(D) OTHER INFORMATION:/note= "X in position 4 is A or E"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Asn Trp Thr Xaa Gly Phe Xaa Xaa Ala Ser  
1 5 10

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Gly Phe Xaa Xaa Ala Ser Xaa Xaa Xaa Xaa Pro  
1 5 10

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 16 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

(A) NAME/KEY: Modified-site  
(B) LOCATION:2  
(D) OTHER INFORMATION:/note= "X in position 2 is N or D"

(ix) FEATURE:

(A) NAME/KEY: Modified-site  
(B) LOCATION:3  
(D) OTHER INFORMATION:/note= "X in position 3 is P or E"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
  - (B) LOCATION:6
  - (D) OTHER INFORMATION:/note= "X in position 6 is T or L"

(ix) FEATURE:

  - (A) NAME/KEY: Modified-site
  - (B) LOCATION:7
  - (D) OTHER INFORMATION:/note= "X in position 7 is W or F"

(ix) FEATURE:

  - (A) NAME/KEY: Modified-site
  - (B) LOCATION:10
  - (D) OTHER INFORMATION:/note= "X in position 10 is S or K"

(ix) FEATURE:

  - (A) NAME/KEY: Modified-site
  - (B) LOCATION:13
  - (D) OTHER INFORMATION:/note= "X in position 13 is V or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Pro Xaa Xaa Xaa Arg Xaa Xaa Xaa Xaa Xaa Xaa Xaa Pro Phe Ser  
 1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 11 amino acids
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (v) FRAGMENT TYPE: internal
  - (ix) FEATURE:
    - (A) NAME/KEY: Modified-site
    - (B) LOCATION:2
    - (D) OTHER INFORMATION:/note= "X in position 2 is Q or A"
  - (ix) FEATURE:
    - (A) NAME/KEY: Modified-site
    - (B) LOCATION:3
    - (D) OTHER INFORMATION:/note= "X in position 3 is V or L"
  - (ix) FEATURE:
    - (A) NAME/KEY: Modified-site
    - (B) LOCATION:11
    - (D) OTHER INFORMATION:/note= "X in position 11 is G or A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Asp Xaa Xaa Gln Pro Leu Xaa Phe Cys Gly Xaa  
1 5 10

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 19 amino acids
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (v) FRAGMENT TYPE: internal
  - (ix) FEATURE:
    - (A) NAME/KEY: Modified-site
    - (B) LOCATION: 7
    - (D) OTHER INFORMATION:/note= "X in position 7 is Y or F"

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION:17
- (D) OTHER INFORMATION:/note= "X in position 17 is E or A"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Phe Val Glu Ser Gln Xaa Xaa Ala Arg Xaa Xaa Gly Xaa Gly Asp Phe  
1                   5                   10                   15  
Xaa Lys Cys

## (2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION:4
- (D) OTHER INFORMATION:/note= "X in position 4 is A or E"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Asn Trp Thr Xaa Gly Phe  
1                   5

## (2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION:3
- (D) OTHER INFORMATION:/note= "X in position 3 is F or Y"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Asp Lys Xaa Tyr Gly Thr  
1                   5

## (2) INFORMATION FOR SEQ ID NO: 12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION:5
- (D) OTHER INFORMATION:/note= "X in position 5 is F or Y"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Asp Leu Asp Lys Xaa Tyr Gly  
1 5

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 5 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION:4
- (D) OTHER INFORMATION:/note= "X in position 4 is A or E"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Gly Asp Phe Xaa Lys  
1 5

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 6 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION:3
- (D) OTHER INFORMATION:/note= "X in position 3 is N or H"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION:4
- (D) OTHER INFORMATION:/note= "X in position 4 is I or L"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Gln Val Xaa Xaa Ile Gln  
1 5

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "sense primer"

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCCAAGCTTA AYTGGACNGM NGGNNTT

26

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "sense primer"

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCCAAGCTTG AYAARTWYGG NAC

23

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "anti-sense primer"

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GCTCTAGACR TARWAYTTRT CNARRTC

27

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "anti-sense primer"

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GCTCTAGACA YTTNKCRAAR TCNCC

25

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "sense primer"

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CCCAAGCTTC ARGTNMAYMT NATHCA

26

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "anti-sense primer"

(iv) ANTI-SENSE: YES

(\*i) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GCTCTAGACR AANCCNKNG TCCARTT

27

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1501 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Agrocybe pediades
- (B) STRAIN: CBS 900.96

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:17..1375

(ix) FEATURE:

- (A) NAME/KEY: sig\_peptide
- (B) LOCATION:17..94

(ix) FEATURE:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION:95..1375

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGATCCGAAT TCACATT ATG TCC CTC TTC ATC GGC GGC TGT TTG CTC GTG	49
Met Ser Leu Phe Ile Gly Gly Cys Leu Leu Val	
-26 -25 -20	

TTT TTA CAG GCG AGC GCA TAC GGC GGC GTC GTG CAG GCC ACA TTC GTG	97
Phe Leu Gln Ala Ser Ala Tyr Gly Gly Val Val Gln Ala Thr Phe Val	
-15 -10 -5 1	

CAG CCG TTT TTC CCT CCA CAG ATT CAG GAC TCT TGG GCA GCT TAT ACA	145
Gln Pro Phe Pro Pro Gln Ile Gln Asp Ser Trp Ala Ala Tyr Thr	
5 10 15	

CCA TAT TAT CCT GTT CAG GCG TAC ACG CCT CCC CCG AAG GAT TGC AAG	193
Pro Tyr Tyr Pro Val Gln Ala Tyr Thr Pro Pro Lys Asp Cys Lys	
20 25 30	

ATC ACA CAA GTT AAC ATT ATT CAA CGA CAT GGT GCC CGC TTT CCG ACA	241
Ile Thr Gln Val Asn Ile Ile Gln Arg His Gly Ala Arg Phe Pro Thr	
35 40 45	

TCG GGG GCA GGC ACA AGG ATC CAA GCA GCT GTG AAG AAG CTT CAA TCA Ser Gly Ala Gly Thr Arg Ile Gln Ala Ala Val Lys Lys Leu Gln Ser 50 55 60 65	289
GCT AAA ACC TAT ACG GAT CCT CGT CTC GAC TTT CTG ACC AAC TAT ACC Ala Lys Thr Tyr Thr Asp Pro Arg Leu Asp Phe Leu Thr Asn Tyr Thr 70 75 80	337
TAT ACC CTT GGT CAC GAC GAT CTC GTA CCG TTT GGA GCG CTT CAA TCA Tyr Thr Leu Gly His Asp Asp Leu Val Pro Phe Gly Ala Leu Gln Ser 85 90 95	385
TCA CAA GCT GGA GAG GAA ACG TTT CAA CGA TAC TCG TTT CTG GTG TCC Ser Gln Ala Gly Glu Glu Thr Phe Gln Arg Tyr Ser Phe Leu Val Ser 100 105 110	433
AAA GAG AAC TTA CCT TTT GTA AGA GCT TCG AGT TCC AAT CGA GTC GTC Lys Glu Asn Leu Pro Phe Val Arg Ala Ser Ser Asn Arg Val Val 115 120 125	481
GAC TCA GCT ACC AAC TGG ACG GAA GGT TTT TCT GCG GCC AGT CAC CAC Asp Ser Ala Thr Asn Trp Thr Glu Gly Phe Ser Ala Ala Ser His His 130 135 140 145	529
GTC TTG AAT CCC ATT CTC TTT GTA ATC CTC TCA GAA AGT CTC AAT GAC Val Leu Asn Pro Ile Leu Phe Val Ile Leu Ser Glu Ser Leu Asn Asp 150 155 160	577
ACG CTT GAC GAT GCC ATG TGC CCT AAC GCG GGC TCC TCC GAC CCG CAG Thr Leu Asp Asp Ala Met Cys Pro Asn Ala Gly Ser Ser Asp Pro Gln 165 170 175	625
ACT GGT ATC TGG ACC TCG ATA TAC GGG ACG CCT ATT GCC AAC CGA CTA Thr Gly Ile Trp Thr Ser Ile Tyr Gly Thr Pro Ile Ala Asn Arg Leu 180 185 190	673
AAT CAG CAG GCT CCG GGT GCA AAT ATT ACA GCT GCC GAT GTG TCG AAC Asn Gln Ala Pro Gly Ala Asn Ile Thr Ala Ala Asp Val Ser Asn 195 200 205	721
CTT ATA CCG CTT TGC GCA TTC GAG ACG ATA GTA AAG GAG ACG CCA AGT Leu Ile Pro Leu Cys Ala Phe Glu Thr Ile Val Lys Glu Thr Pro Ser 210 215 220 225	769
CCT TTC TGT AAT TTG TTC ACC CCC GAA GAG TTC GCA CAG TTT GAA TAT Pro Phe Cys Asn Leu Phe Thr Pro Glu Glu Phe Ala Gln Phe Glu Tyr 230 235 240	817
TTC GGT GAC CTG GAC AAG TTC TAT GGG ACA GGT TAT GGA CAA CCG TTA Phe Gly Asp Leu Asp Lys Phe Tyr Gly Thr Gly Tyr Gly Gln Pro Leu 245 250 255	865
GGA CCT GTG CAA GGT GTC GGC TAC ATC AAT GAA CTT CTT GCC CGA CTC Gly Pro Val Gln Gly Val Gly Tyr Ile Asn Glu Leu Leu Ala Arg Leu 260 265 270	913
ACA GAA ATG CCA GTT CGA GAT AAC ACC CAG ACG AAC AGG ACA CTC GAC Thr Glu Met Pro Val Arg Asp Asn Thr Gln Thr Asn Arg Thr Leu Asp 275 280 285	961
TCT TCT CCG CTT ACA TTT CCC CTC GAC CGC AGT ATC TAC GCT GAC CTC Ser Ser Pro Leu Thr Phe Pro Leu Asp Arg Ser Ile Tyr Ala Asp Leu 290 295 300 305	1009
TCG CAC GAT AAC CAA ATG ATC GCG ATA TTT TCA GCG ATG GGT CTT TTC Ser His Asp Asn Gln Met Ile Ala Ile Phe Ser Ala Met Gly Leu Phe 310 315 320	1057
AAC CAG AGT TCA CCT TTG GAT CCG TCC TTC CCC AAC CCC AAG CGT ACT Asn Gln Ser S r Pro Leu Asp Pro Ser Phe Pro Asn Pro Lys Arg Thr 325 330 335	1105
TGG GTC ACC AGT CGG CTT ACG CCT TTC AGC GCG AGA ATG GTC ACT GAG Trp Val Thr Ser Arg Leu Thr Pro Phe Ser Ala Arg Met Val Thr Glu 340 345 350	1153

CGG TTG CTG TGT CAA AGG GAT GGG ACA GGG AGC GGT GGA CCA TCC AGG Arg Leu Leu Cys Gln Arg Asp Gly Thr Gly Ser Gly Gly Pro Ser Arg 355 360 365	1201
ATC ATG CGG AAT GGA AAT GTG CAG ACG TTT GTG AGG ATT CTT GTC AAC Ile Met Arg Asn Gly Asn Val Gln Thr Phe Val Arg Ile Leu Val Asn 370 375 380 385	1249
GAT GCT TTA CAG CCT TTG AAG TTC TGC GGA GGG GAC ATG GAT AGT TTG Asp Ala Leu Gln Pro Leu Lys Phe Cys Gly Gly Asp Met Asp Ser Leu 390 395 400	1297
TGT ACT CTG GAA GCG TTC GTC GAG AGC CAG AAG TAT GCA CGA GAG GAT Cys Thr Leu Glu Ala Phe Val Glu Ser Gln Lys Tyr Ala Arg Glu Asp 405 410 415	1345
GGT CAA GGC GAT TTT GAA AAA TGT TTT GAT TAAATATTGC AGTATGCTCA Gly Gln Gly Asp Phe Glu Lys Cys Phe Asp 420 425	1395
GTGAGTAGAC TACAGTGCAG GCCCTGTAAC TCTTGTATTG TGTTTCTGGA ATTCCCTCGGA GCGTAGTTG TAGCAAAAAA AAAAAAAA AAATTCCCTGC GGCCGC	1455
	1501

## (2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 453 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

```

Met Ser Leu Phe Ile Gly Gly Cys Leu Leu Val Phe Leu Gln Ala Ser
-26 -25           -20           -15

Ala Tyr Gly Gly Val Val Gln Ala Thr Phe Val Gln Pro Phe Phe Pro
-10           -5           1           5

Pro Gln Ile Gln Asp Ser Trp Ala Ala Tyr Thr Pro Tyr Tyr Pro Val
10            15           20

Gln Ala Tyr Thr Pro Pro Pro Lys Asp Cys Lys Ile Thr Gln Val Asn
25            30           35

Ile Ile Gln Arg His Gly Ala Arg Phe Pro Thr Ser Gly Ala Gly Thr
40            45           50

Arg Ile Gln Ala Ala Val Lys Lys Leu Gln Ser Ala Lys Thr Tyr Thr
55            60           65           70

Asp Pro Arg Leu Asp Phe Leu Thr Asn Tyr Thr Tyr Thr Leu Gly His
75            80           85

Asp Asp Leu Val Pro Phe Gly Ala Leu Gln Ser Ser Gln Ala Gly Glu
90            95           100

Glu Thr Phe Gln Arg Tyr Ser Phe Leu Val Ser Lys Glu Asn Leu Pro
105          110           115

Phe Val Arg Ala Ser Ser Ser Asn Arg Val Val Asp Ser Ala Thr Asn
120          125           130

Trp Thr Glu Gly Phe Ser Ala Ala Ser His His Val Leu Asn Pro Ile
135          140           145           150

Leu Phe Val Ile Leu Ser Glu Ser Leu Asn Asp Thr Leu Asp Asp Ala
155          160           165

Met Cys Pro Asn Ala Gly Ser Ser Asp Pro Gln Thr Gly Ile Trp Thr
170          175           180

Ser Ile Tyr Gly Thr Pro Ile Ala Asn Arg Leu Asn Gln Gln Ala Pro
185          190           195
  
```

Gly Ala Asn Ile Thr Ala Ala Asp Val Ser Asn Leu Ile Pro Leu Cys  
 200 205 210

Ala Phe Glu Thr Ile Val Lys Glu Thr Pro Ser Pro Phe Cys Asn Leu  
 215 220 225 230

Phe Thr Pro Glu Glu Phe Ala Gln Phe Glu Tyr Phe Gly Asp Leu Asp  
 235 240 245

Lys Phe Tyr Gly Thr Gly Tyr Gly Gln Pro Leu Gly Pro Val Gln Gly  
 250 255 260

Val Gly Tyr Ile Asn Glu Leu Leu Ala Arg Leu Thr Glu Met Pro Val  
 265 270 275

Arg Asp Asn Thr Gln Thr Asn Arg Thr Leu Asp Ser Ser Pro Leu Thr  
 280 285 290

Phe Pro Leu Asp Arg Ser Ile Tyr Ala Asp Leu Ser His Asp Asn Gln  
 295 300 305 310

Met Ile Ala Ile Phe Ser Ala Met Gly Leu Phe Asn Gln Ser Ser Pro  
 315 320 325

Leu Asp Pro Ser Phe Pro Asn Pro Lys Arg Thr Trp Val Thr Ser Arg  
 330 335 340

Leu Thr Pro Phe Ser Ala Arg Met Val Thr Glu Arg Leu Leu Cys Gln  
 345 350 355

Arg Asp Gly Thr Gly Ser Gly Gly Pro Ser Arg Ile Met Arg Asn Gly  
 360 365 370

Asn Val Gln Thr Phe Val Arg Ile Leu Val Asn Asp Ala Leu Gln Pro  
 375 380 385 390

Leu Lys Phe Cys Gly Gly Asp Met Asp Ser Leu Cys Thr Leu Glu Ala  
 395 400 405

Phe Val Glu Ser Gln Lys Tyr Ala Arg Glu Asp Gly Gln Gly Asp Phe  
 410 415 420

Glu Lys Cys Phe Asp  
 425

## (2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1593 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Peniophora lycii
  - (B) STRAIN: CBS 686.96

- (ix) FEATURE:
  - (A) NAME/KEY: sig\_peptide
  - (B) LOCATION: 123..212

- (ix) FEATURE:
  - (A) NAME/KEY: mat\_peptide
  - (B) LOCATION: 213..1439

- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 123..1439

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GGATCCGAAT TCCATCTTCT GCTCTGACCT CCATCTCGCT GAGCGGCCGA CGAGAACCTA	60
GGGGCTCTAA GTCCACGTAC TATCGCCGCG CCTGTGAAGG CCCCATACCA GCCCTTATCG	120

AT ATG GTT TCT TCG GCA TTC GCA CCT TCC ATC CTA CTT AGC TTG ATG Met Val Ser Ser Ala Phe Ala Pro Ser Ile Leu Leu Ser Leu Met -30 -25 -20	167
TCG AGT CTT GCT TTG AGC ACG CAG TTC AGC TTT GTT GCG GCG CAG CTA Ser Ser Leu Ala Leu Ser Thr Gln Phe Ser Phe Val Ala Ala Gln Leu -15 -10 -5 1	215
CCT ATC CCC GCA CAA AAC ACA AGT AAT TGG GGG CCT TAC GAT CCC TTC Pro Ile Pro Ala Gln Asn Thr Ser Asn Trp Gly Pro Tyr Asp Pro Phe 5 10 15	263
TTT CCC GTC GAA CCG TAT GCA GCT CCG CCG GAA GGG TGC ACA GTG ACA Phe Pro Val Glu Pro Tyr Ala Ala Pro Pro Glu Gly Cys Thr Val Thr 20 25 30	311
CAG GTC AAC CTG ATT CAG AGG CAC GGC GCG CGT TGG CCC ACA TCC GGC Gln Val Asn Leu Ile Gln Arg His Gly Ala Arg Trp Pro Thr Ser Gly 35 40 45	359
GCG CGG TCG CGG CAG GTC GCC GCC GTA GCG AAG ATA CAA ATG GCG CGA Ala Arg Ser Arg Gln Val Ala Ala Val Ala Lys Ile Gln Met Ala Arg 50 55 60 65	407
CCA TTC ACG GAT CCC AAG TAT GAG TTC CTC AAC GAC TTC GTG TAC AAG Pro Phe Thr Asp Pro Lys Tyr Glu Phe Leu Asn Asp Phe Val Tyr Lys 70 75 80	455
TTC GGC GTC GCC GAT CTG CTA CCG TTC GGG GCT AAC CAA TCG CAC CAA Phe Gly Val Ala Asp Leu Leu Pro Phe Gly Ala Asn Gln Ser His Gln 85 90 95	503
ACC GGC ACC GAT ATG TAT ACG CGC TAC AGT ACA CTA TTT GAG GGC GGG Thr Gly Thr Asp Met Tyr Thr Arg Tyr Ser Thr Leu Phe Glu Gly Gly 100 105 110	551
GAT GTA CCC TTT GTG CGC GCG GCT GGT GAC CAA CGC GTC GTT GAC TCC Asp Val Pro Phe Val Arg Ala Ala Gly Asp Gln Arg Val Val Asp Ser 115 120 125	599
TCG ACG AAC TGG ACG GCA GGC TTT GGC GAT GCT TCT GGC GAG ACT GTT Ser Thr Asn Trp Thr Ala Gly Phe Gly Asp Ala Ser Gly Glu Thr Val 130 135 140 145	647
CTC CCG ACG CTC CAG GTT GTG CTT CAA GAA GAG GGG AAC TGC ACG CTC Leu Pro Thr Leu Gln Val Val Leu Gln Glu Glu Gly Asn Cys Thr Leu 150 155 160	695
TGC AAT AAT ATG TGC CCG AAT GAA GTG GAT GGT GAC GAA TCC ACA ACG Cys Asn Asn Met Cys Pro Asn Glu Val Asp Gly Asp Glu Ser Thr Thr 165 170 175	743
TGG CTG GGG GTC TTT GCG CCG AAC ATC ACC GCG CGA TTG AAC GCT GCT Trp Leu Gly Val Phe Ala Pro Asn Ile Thr Ala Arg Leu Asn Ala Ala 180 185 190	791
GCG CCG AGT GCC AAC CTC TCA GAC AGC GAC GCG CTC ACT CTC ATG GAT Ala Pro Ser Ala Asn Leu Ser Asp Ser Asp Ala Leu Thr Leu Met Asp 195 200 205	839
ATG TGC CCG TTC GAC ACT CTC AGC TCC GGG AAC GCC AGC CCC TTC TGT Met Cys Pro Phe Asp Thr Leu Ser Ser Gly Asn Ala Ser Pro Phe Cys 210 215 220 225	887
GAC CTA TTT ACC GCG GAG GAG TAT GTG TCG TAC GAG TAC TAC TAT GAC Asp Leu Phe Thr Ala Glu Glu Tyr Val Ser Tyr Glu Tyr Tyr Tyr Asp 230 235 240	935
CTC GAC AAG TAC TAT GGC ACG GGC CCC GGG AAC GCT CTC GGT CCT GTC Leu Asp Lys Tyr Tyr Gly Thr Gly Pro Gly Asn Ala Leu Gly Pro Val 245 250 255	983
CAG GGC GTC GGA TAC GTC AAT GAG CTG CTT GCA CGC TTG ACC GGC CAA Gln Gly Val Gly Tyr Val Asn Glu Leu Leu Ala Arg Leu Thr Gly Gln 260 265 270	1031

GCC GTT CGA GAC GAG ACG CAG ACG AAC CGC ACG CTC GAC AGC GAC CCT Ala Val Arg Asp Glu Thr Gln Thr Asn Arg Thr Leu Asp Ser Asp Pro 275 280 285	1079
GCA ACA TTC CCG CTG AAC CGT ACG TTC TAC GCC GAC TTC TCG CAT GAT Ala Thr Phe Pro Leu Asn Arg Thr Phe Tyr Ala Asp Phe Ser His Asp 290 295 300 305	1127
AAC ACC ATG GTG CCC ATC TTT GCG GCG CTC GGG CTC TTC AAC GCC ACC Asn Thr Met Val Pro Ile Phe Ala Ala Leu Gly Leu Phe Asn Ala Thr 310 315 320	1175
GCC CTC GAC CCG CTG AAG CCC GAC GAG AAC AGG TTG TGG GTG GAC TCT Ala Leu Asp Pro Leu Lys Pro Asp Glu Asn Arg Leu Trp Val Asp Ser 325 330 335	1223
AAG CTG GTA CCG TTC TCT GGA CAT ATG ACG GTC GAG AAG CTG GCA TGT Lys Leu Val Pro Phe Ser Gly His Met Thr Val Glu Lys Leu Ala Cys 340 345 350	1271
TCT GGG AAG GAG GCG GTC AGG GTG CTC GTG AAC GAC GCG GTG CAG CCG Ser Gly Lys Glu Ala Val Arg Val Leu Val Asn Asp Ala Val Gln Pro 355 360 365	1319
CTG GAG TTC TGC GGA GGT GTT GAT GGG GTG TGC GAG CTT TCG GCT TTC Leu Glu Phe Cys Gly Val Asp Gly Val Cys Glu Leu Ser Ala Phe 370 375 380 385	1367
GTA GAG AGC CAG ACG TAT GCG CGG GAG AAT GGG CAA GGC GAC TTC GCC Val Glu Ser Gln Thr Tyr Ala Arg Glu Asn Gly Gln Gly Asp Phe Ala 390 395 400	1415
AAG TGC GGC TTT GTT CCG TCG GAA TAGCGGGAGA CCGTCTATGC TACACAGTAA Lys Cys Gly Phe Val Pro Ser Glu 405	1469
TTGTGTACTC TATAGCACTG TAGCTGTACT TACAAGTCGT AGGGTACGAT CGTACTTACG	1529
CTCGTTTATT GATCCTTCCT TTAAAAAAA AAAAAAAA AAAAAAAA ATTCCCTGCGG	1589
CCGC	1593

## (2) INFORMATION FOR SEQ ID NO: 24:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 439 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Met Val Ser Ser Ala Phe Ala Pro Ser Ile Leu Leu Ser Leu Met Ser  
-30 -25 -20 -15

Ser Leu Ala Leu Ser Thr Gln Phe Ser Phe Val Ala Ala Gln Leu Pro  
-10 -5 1

Ile Pro Ala Gln Asn Thr Ser Asn Trp Gly Pro Tyr Asp Pro Phe Phe  
5 10 15

Pro Val Glu Pro Tyr Ala Ala Pro Pro Glu Gly Cys Thr Val Thr Gln  
20 25 30

Val Asn Leu Ile Gln Arg His Gly Ala Arg Trp Pro Thr Ser Gly Ala  
35 40 45 50

Arg Ser Arg Gln Val Ala Ala Val Ala Lys Ile Gln Met Ala Arg Pro  
55 60 65

Phe Thr Asp Pro Lys Tyr Glu Phe Leu Asn Asp Phe Val Tyr Lys Phe  
70 75 80

Gly Val Ala Asp Leu Leu Pro Phe Gly Ala Asn Gln Ser His Gln Thr  
85 90 95

120

Gly Thr Asp Met Tyr Thr Arg Tyr Ser Thr Leu Phe Glu Gly Gly Asp  
 100 105 110

Val Pro Phe Val Arg Ala Ala Gly Asp Gln Arg Val Val Asp Ser Ser  
 115 120 125 130

Thr Asn Trp Thr Ala Gly Phe Gly Asp Ala Ser Gly Glu Thr Val Leu  
 135 140 145

Pro Thr Leu Gln Val Val Leu Gln Glu Glu Gly Asn Cys Thr Leu Cys  
 150 155 160

Asn Asn Met Cys Pro Asn Glu Val Asp Gly Asp Glu Ser Thr Thr Trp  
 165 170 175

Leu Gly Val Phe Ala Pro Asn Ile Thr Ala Arg Leu Asn Ala Ala Ala  
 180 185 190

Pro Ser Ala Asn Leu Ser Asp Ser Asp Ala Leu Thr Leu Met Asp Met  
 195 200 205 210

Cys Pro Phe Asp Thr Leu Ser Ser Gly Asn Ala Ser Pro Phe Cys Asp  
 215 220 225

Leu Phe Thr Ala Glu Glu Tyr Val Ser Tyr Glu Tyr Tyr Asp Leu  
 230 235 240

Asp Lys Tyr Tyr Gly Thr Gly Pro Gly Asn Ala Leu Gly Pro Val Gln  
 245 250 255

Gly Val Gly Tyr Val Asn Glu Leu Leu Ala Arg Leu Thr Gly Gln Ala  
 260 265 270

Val Arg Asp Glu Thr Gln Thr Asn Arg Thr Leu Asp Ser Asp Pro Ala  
 275 280 285 290

Thr Phe Pro Leu Asn Arg Thr Phe Tyr Ala Asp Phe Ser His Asp Asn  
 295 300 305

Thr Met Val Pro Ile Phe Ala Ala Leu Gly Leu Phe Asn Ala Thr Ala  
 310 315 320

Leu Asp Pro Leu Lys Pro Asp Glu Asn Arg Leu Trp Val Asp Ser Lys  
 325 330 335

Leu Val Pro Phe Ser Gly His Met Thr Val Glu Lys Leu Ala Cys Ser  
 340 345 350

Gly Lys Glu Ala Val Arg Val Leu Val Asn Asp Ala Val Gln Pro Leu  
 355 360 365 370

Glu Phe Cys Gly Gly Val Asp Gly Val Cys Glu Leu Ser Ala Phe Val  
 375 380 385

Glu Ser Gln Thr Tyr Ala Arg Glu Asn Gly Gln Gly Asp Phe Ala Lys  
 390 395 400

Cys Gly Phe Val Pro Ser Glu  
 405

## (2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1522 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Paxillus involutus
  - (B) STRAIN: CBS 100231
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 58..1383

CTG TTC GAG GGC ATC CCT GGC TCT TTC GAG GCG TTC GCC TAT GGT GGC Leu Phe Glu Gly Ile Pro Gly Ser Phe Glu Ala Phe Ala Tyr Gly Gly 240 245 250	873
GAC CTT GAC AAG TTC TAC GGT ACC GGT TAC GGT CAG GAA CTC GGA CCC Asp Leu Asp Lys Phe Tyr Gly Thr Gly Tyr Gly Gln Glu Leu Gly Pro 255 260 265	921
GTT CAA GGC GTC GGC TAC GTC AAC GAG CTC ATC GCC CGC CTC ACC AAC Val Gln Gly Val Gly Tyr Val Asn Glu Leu Ile Ala Arg Leu Thr Asn 270 275 280 285	969
TCC GCC GTC CGC GAC AAC ACC CAG ACG AAC CGC ACA CTC GAC GCC TCG Ser Ala Val Arg Asp Asn Thr Gln Thr Asn Arg Thr Leu Asp Ala Ser 290 295 300	1017
CCC GTA ACC TTC CCG TTG AAC AAG ACG TTC TAC GCC GAT TTC TCC CAC Pro Val Thr Phe Pro Leu Asn Lys Thr Phe Tyr Ala Asp Phe Ser His 305 310 315	1065
GAC AAC CTC ATG GTC GCC GTC TTC TCC GCC ATG GGC CTC TTC CGC CAG Asp Asn Leu Met Val Ala Val Phe Ser Ala Met Gly Leu Phe Arg Gln 320 325 330	1113
CCC GCG CCG CTC AGC ACG TCC GTG CCG AAC CCA TGG CGC ACG TGG CGC Pro Ala Pro Leu Ser Thr Ser Val Pro Asn Pro Trp Arg Thr Trp Arg 335 340 345	1161
ACG AGC TCC CTC GTC CCC TTC TCC GGA CGC ATG GTC GTG GAA CGC CTC Thr Ser Ser Leu Val Pro Phe Ser Gly Arg Met Val Val Glu Arg Leu 350 355 360 365	1209
AGC TGT TTC GGC ACG ACC AAG GTT CGC GTC CTC GTG CAG GAC CAG GTG Ser Cys Phe Gly Thr Thr Lys Val Arg Val Leu Val Gln Asp Gln Val 370 375 380	1257
CAG CCG CTC GAG TTC TGC GGG GGT GAT AGG AAC GGG CTG TGC ACG CTT Gln Pro Leu Glu Phe Cys Gly Gly Asp Arg Asn Gly Leu Cys Thr Leu 385 390 395	1305
GCT AAG TTT GTG GAG AGC CAG ACG TTT GCG AGG AGT GAT GGT GCG GGG Ala Lys Phe Val Glu Ser Gln Thr Phe Ala Arg Ser Asp Gly Ala Gly 400 405 410	1353
GAC TTT GAG AAG TGC TTC GCG ACC TCG GCG TGAGGATGGA CGAACAAAAT Asp Phe Glu Lys Cys Phe Ala Thr Ser Ala 415 420	1403
TAAATTGGGG TATTTTATCG TATAATTATG GTGTGTGTAG AACATGGGCT CGGGGTCGAT GGTGAAAAGC AAAGGTTTAT CGTCTAAAAA AAAAAAAA AAAAATTCC TGC GGCCGC	1463 1522
(2) INFORMATION FOR SEQ ID NO: 26:	
(i) SEQUENCE CHARACTERISTICS:	
(A) LENGTH: 442 amino acids	
(B) TYPE: amino acid	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: protein	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:	
Met Leu Phe Gly Phe Val Ala Leu Ala Cys Leu Leu Ser Leu Ser Glu -19 -15 -10 -5	
Val Leu Ala Thr Ser Val Pro Lys Asn Thr Ala Pro Thr Phe Pro Ile 1 5 10	
Pro Glu Ser Glu Gln Arg Asn Trp Ser Pro Tyr Ser Pro Tyr Phe Pro 15 20 25	
Leu Ala Glu Tyr Lys Ala Pro Pro Ala Gly Cys Gln Ile Asn Gln Val 30 35 40 45	

## (ix) FEATURE:

(A) NAME/KEY: mat\_peptide  
 (B) LOCATION: 115..1383

## (ix) FEATURE:

(A) NAME/KEY: sig\_peptide  
 (B) LOCATION: 58..114

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GGATCCGAAT TCGGCACTCG TACGGTCCCC CGGTCTACCC TCTGCTCGCC TTGGAAG	57
ATG CTC TTC GGT TTC GTC GCC CTC GCC TGT CTC TTG TCC CTC TCC TCC GAG Met Leu Phe Gly Phe Val Ala Leu Ala Cys Leu Leu Ser Leu Ser Glu -19 -15 -10 -5	105
GTC CTT GCG ACC TCC GTG CCC AAG AAC ACA GCG CCG ACC TTC CCC ATT Val Leu Ala Thr Ser Val Pro Lys Asn Thr Ala Pro Thr Phe Pro Ile 1 5 10	153
CCG GAG AGT GAG CAG CGG AAC TGG TCC CCG TAC TCG CCC TAC TTC CCT Pro Glu Ser Glu Gln Arg Asn Trp Ser Pro Tyr Ser Pro Tyr Phe Pro 15 20 25	201
CTT GCC GAG TAC AAG GCT CCT CCG GCG GGC TGC CAG ATC AAC CAG GTC Leu Ala Glu Tyr Lys Ala Pro Pro Ala Gly Cys Gln Ile Asn Gln Val 30 35 40 45	249
AAC ATC ATC CAA AGA CAT GGT GCC CGG TTC CCG ACC TCT GGC GCG ACC Asn Ile Ile Gln Arg His Gly Ala Arg Phe Pro Thr Ser Gly Ala Thr 50 55 60	297
ACC CGT ATC AAG GCG GGT TTG ACC AAG TTG CAA GGC GTC CAG AAC TTT Thr Arg Ile Lys Ala Gly Leu Thr Lys Leu Gln Gly Val Gln Asn Phe 65 70 75	345
ACC GAC GCC AAA TTC AAC TTC ATC AAG TCG TTC AAG TAC GAT CTC GGT Thr Asp Ala Lys Phe Asn Phe Ile Lys Ser Phe Lys Tyr Asp Leu Gly 80 85 90	393
AAC TCG GAC CTC GTT CCG TTC GGT GCA GCA CAG TCC TTC GAC GCT GGT Asn Ser Asp Leu Val Pro Phe Gly Ala Ala Gln Ser Phe Asp Ala Gly 95 100 105	441
CAG GAG GCC TTC GCC CGC TAC TCG AAG CTT GTC AGC AAG AAC AAC CTG Gln Glu Ala Phe Ala Arg Tyr Ser Lys Leu Val Ser Lys Asn Asn Leu 110 115 120 125	489
CCG TTC ATT CGT GCC GAT GGA AGT GAT CGT GTT GTG GAT TCT GCT ACA Pro Phe Ile Arg Ala Asp Gly Ser Asp Arg Val Val Asp Ser Ala Thr 130 135 140	537
AAC TGG ACT GCG GGT TTC GCT TCG GCA AGT CAC AAC ACG GTC CAG CCC Asn Trp Thr Ala Gly Phe Ala Ser Ala Ser His Asn Thr Val Gln Pro 145 150 155	585
AAG CTG AAC CTG ATT CTC CCG CAA ACT GGC AAT GAT ACC CTG GAA GAT Lys Leu Asn Leu Ile Leu Pro Gln Thr Gly Asn Asp Thr Leu Glu Asp 160 165 170	633
AAT ATG TGC CCT GCT GCT GGC GAT TCT GAC CCC CAG GTC AAC GCG TGG Asn Met Cys Pro Ala Ala Gly Asp Ser Asp Pro Gln Val Asn Ala Trp 175 180 185	681
TTG GCT GTT GCT TTC CCT TCC ATC ACT GCA CGG CTC AAC GCC GCG GCG Leu Ala Val Ala Phe Pro Ser Ile Thr Ala Arg Leu Asn Ala Ala Ala 190 195 200 205	729
CCC TCT GTC AAC CTC ACC GAC ACG GAC GCG TTC AAC CTC GTC AGT CTC Pro Ser Val Asn Leu Thr Asp Thr Asp Ala Phe Asn Leu Val Ser Leu 210 215 220	777
TGC GCT TTC TTG ACA GTC TCG AAG GAG AAG AAG ÁGT GAC TTC TGC ACC Cys Ala Phe Leu Thr Val Ser Lys Glu Lys Lys Ser Asp Phe Cys Thr 225 230 235	825

Asn Ile Ile Gln Arg His Gly Ala Arg Phe Pro Thr Ser Gly Ala Thr  
 50 55 60  
 Thr Arg Ile Lys Ala Gly Leu Thr Lys Leu Gln Gly Val Gln Asn Phe  
 65 70 75  
 Thr Asp Ala Lys Phe Asn Phe Ile Lys Ser Phe Lys Tyr Asp Leu Gly  
 80 85 90  
 Asn Ser Asp Leu Val Pro Phe Gly Ala Ala Gln Ser Phe Asp Ala Gly  
 95 100 105  
 Gln Glu Ala Phe Ala Arg Tyr Ser Lys Leu Val Ser Lys Asn Asn Leu  
 110 115 120 125  
 Pro Phe Ile Arg Ala Asp Gly Ser Asp Arg Val Val Asp Ser Ala Thr  
 130 135 140  
 Asn Trp Thr Ala Gly Phe Ala Ser Ala Ser His Asn Thr Val Gln Pro  
 145 150 155  
 Lys Leu Asn Leu Ile Leu Pro Gln Thr Gly Asn Asp Thr Leu Glu Asp  
 160 165 170  
 Asn Met Cys Pro Ala Ala Gly Asp Ser Asp Pro Gln Val Asn Ala Trp  
 175 180 185  
 Leu Ala Val Ala Phe Pro Ser Ile Thr Ala Arg Leu Asn Ala Ala Ala  
 190 195 200 205  
 Pro Ser Val Asn Leu Thr Asp Thr Asp Ala Phe Asn Leu Val Ser Leu  
 210 215 220  
 Cys Ala Phe Leu Thr Val Ser Lys Glu Lys Lys Ser Asp Phe Cys Thr  
 225 230 235  
 Leu Phe Glu Gly Ile Pro Gly Ser Phe Glu Ala Phe Ala Tyr Gly Gly  
 240 245 250  
 Asp Leu Asp Lys Phe Tyr Gly Thr Gly Tyr Gly Gln Glu Leu Gly Pro  
 255 260 265  
 Val Gln Gly Val Gly Tyr Val Asn Glu Leu Ile Ala Arg Leu Thr Asn  
 270 275 280 285  
 Ser Ala Val Arg Asp Asn Thr Gln Thr Asn Arg Thr Leu Asp Ala Ser  
 290 295 300  
 Pro Val Thr Phe Pro Leu Asn Lys Thr Phe Tyr Ala Asp Phe Ser His  
 305 310 315  
 Asp Asn Leu Met Val Ala Val Phe Ser Ala Met Gly Leu Phe Arg Gln  
 320 325 330  
 Pro Ala Pro Leu Ser Thr Ser Val Pro Asn Pro Trp Arg Thr Trp Arg  
 335 340 345  
 Thr Ser Ser Leu Val Pro Phe Ser Gly Arg Met Val Val Glu Arg Leu  
 350 355 360 365  
 Ser Cys Phe Gly Thr Thr Lys Val Arg Val Leu Val Gln Asp Gln Val  
 370 375 380  
 Gln Pro Leu Glu Phe Cys Gly Asp Arg Asn Gly Leu Cys Thr Leu  
 385 390 395  
 Ala Lys Phe Val Glu Ser Gln Thr Phe Ala Arg Ser Asp Gly Ala Gly  
 400 405 410  
 Asp Phe Glu Lys Cys Phe Ala Thr Ser Ala  
 415 420

(2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1642 base pairs

(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: lin ar

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Paxillus involutus  
 (B) STRAIN: CBS 100231

(ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 48..1373

(ix) FEATURE:

(A) NAME/KEY: mat\_peptide  
 (B) LOCATION: 105..1373

(ix) FEATURE:

(A) NAME/KEY: sig\_peptide  
 (B) LOCATION: 48..104

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GGATCCGAAT TCCAGTCCCC AAGCTAACCC TCTGCTCGCC TTGGAAG ATG CAC CTC Met His Leu -19	56
GGC TTC GTC ACC CTC GCT TGT CTC ATA CAC CTC TCC GAG GTC TTC GCG Gly Phe Val Thr Leu Ala Cys Leu Ile His Leu Ser Glu Val Phe Ala -15 -10 -5	104
GCA TCC GTG CCC CGG AAT ATT GCT CCG AAG TTC TCA ATT CCG GAA AGC Ala Ser Val Pro Arg Asn Ile Ala Pro Lys Phe Ser Ile Pro Glu Ser 1 5 10 15	152
GAG CAG CGA AAC TGG TCG CCT TAC TCT CCT TAC TTT CCC CTA GCC GAA Glu Gln Arg Asn Trp Ser Pro Tyr Ser Pro Tyr Phe Pro Leu Ala Glu 20 25 30	200
TAC AAG GCT CCT CCA GCA GGC TGC GAG ATT AAC CAA GTC AAT ATT ATC Tyr Lys Ala Pro Pro Ala Gly Cys Glu Ile Asn Gln Val Asn Ile Ile 35 40 45	248
CAA CGG CAT GGC GCA CGG TTC CCA ACC TCG GGT GCG GCC ACT CGC ATC Gln Arg His Gly Ala Arg Phe Pro Thr Ser Gly Ala Ala Thr Arg Ile 50 55 60	296
AAG GCT GGT TTA AGC AAG CTG CAA TCC GTC CAG AAT TTC ACC GAC CCC Lys Ala Gly Leu Ser Lys Leu Gln Ser Val Gln Asn Phe Thr Asp Pro 65 70 75 80	344
AAA TTC GAC TTC ATC AAG TCG TTC ACA TAC GAT CTT GGT ACT TCC GAC Lys Phe Asp Phe Ile Lys Ser Phe Thr Tyr Asp Leu Gly Thr Ser Asp 85 90 95	392
CTC GTG CCA TTC GGC GCA GCA CAA TCA TTC GAT GCC GGC CTG GAG GTC Leu Val Pro Phe Gly Ala Ala Gln Ser Phe Asp Ala Gly Leu Glu Val 100 105 110	440
TTC GCT CGC TAT TCG AAG CTC GTC AGC TCG GAC AAC CTG CCT TTC ATT Phe Ala Arg Tyr Ser Lys Leu Val Ser Ser Asp Asn Leu Pro Phe Ile 115 120 125	488
CGC TCA GAT GGT AGC GAT CGT GTA GTC GAC ACT GCT ACG AAC TGG ACT Arg Ser Asp Gly Ser Asp Arg Val Val Asp Thr Ala Thr Asn Trp Thr 130 135 140	536
GCA GGT TTT GCT TCC GCG AGC AAC GCG ATC CAA CCC AAG CTC GAC Ala Gly Phe Ala Ser Ala Ser Arg Asn Ala Ile Gln Pro Lys Leu Asp 145 150 155 160	584
TTG ATA CTT CCA CAA ACT GGC AAT GAC ACC CTC GAG GAC AAC ATG TGT Leu Ile Leu Pro Gln Thr Gly Asn Asp Thr Leu Glu Asp Asn Met Cys 165 170 175	632

CCA GCT GCT GGC GAA TCC GAC CCT CAG GTC GAT GCG TGG TTG GCG TCC Pro Ala Ala Gly Glu Ser Asp Pro Gln Val Asp Ala Trp Leu Ala Ser 180 185 190	680
GCC TTC CCA TCT GTC ACC GCG CAG CTC AAC GCT GCA GCG CCT GGT GCC Ala Phe Pro Ser Val Thr Ala Gln Leu Asn Ala Ala Pro Gly Ala 195 200 205	728
AAT CTC ACA GAC GCC GAC GCC TTC AAC CTC GTC AGC CTG TGT CCC TTC Asn Leu Thr Asp Ala Asp Ala Phe Asn Leu Val Ser Leu Cys Pro Phe 210 215 220	776
ATG ACA GTT TCG AAG GAG CAG AAG AGC GAC TTC TGC ACG TTG TTC GAG Met Thr Val Ser Lys Glu Gln Lys Ser Asp Phe Cys Thr Leu Phe Glu 225 230 235 240	824
GGA ATC CCT GGA TCG TTC GAG GCG TTT GCC TAT GCC GGC GAC CTT GAC Gly Ile Pro Gly Ser Phe Glu Ala Phe Ala Tyr Ala Gly Asp Leu Asp 245 250 255	872
AAG TTC TAT GGG ACC GGC TAT GGC CAA GCC CTC GGA CCG GTC CAA GGC Lys Phe Tyr Gly Thr Gly Gln Ala Leu Gly Pro Val Gln Gly 260 265 270	920
GTC GGC TAC ATC AAC GAG CTC CTT GCA CGC CTG ACC AAC TCC GCA GTG Val Gly Tyr Ile Asn Glu Leu Ala Arg Leu Thr Asn Ser Ala Val 275 280 285	968
AAC GAC AAC ACA CAG ACG AAC CGC ACA CTC GAC GCC GCA CCA GAC ACG Asn Asp Asn Thr Gln Thr Asn Arg Thr Leu Asp Ala Ala Pro Asp Thr 290 295 300	1016
TTC CCG CTC AAC AAG ACC ATG TAC GCC GAT TTC TCA CAC GAC AAC CTC Phe Pro Leu Asn Lys Thr Met Tyr Ala Asp Phe Ser His Asp Asn Leu 305 310 315 320	1064
ATG GTC GCC GTG TTC TCC GCC ATG GGC CTC TTC CGC CAA TCC GCA CCG Met Val Ala Val Phe Ser Ala Met Gly Leu Phe Arg Gln Ser Ala Pro 325 330 335	1112
CTC AGC ACG TCC ACA CCG GAT CCG AAC CGC ACG TGG CTC ACG AGC TCT Leu Ser Thr Ser Thr Pro Asp Pro Asn Arg Thr Trp Leu Thr Ser Ser 340 345 350	1160
GTC GTT CCG TTC TCC GCG CGC ATG GCC GTC GAA CGC CTC AGC TGT GCT Val Val Pro Phe Ser Ala Arg Met Ala Val Glu Arg Leu Ser Cys Ala 355 360 365	1208
GGT ACC ACG AAG GTG CGC GTC CTG GTG CAG GAC CAG GTC CAG CCA CTC Gly Thr Thr Lys Val Arg Val Leu Val Gln Asp Gln Val Gln Pro Leu 370 375 380	1256
GAG TTC TGC GGC GGC GAC CAG GAT GGG TTG TGC GCG CTA GAC AAG TTC Glu Phe Cys Gly Gly Asp Gln Asp Gly Leu Cys Ala Leu Asp Lys Phe 385 390 395 400	1304
GTC GAG AGC CAG GCG TAT GCA CGG AGT GGT GGC GCA GGT GAC TTT GAG Val Glu Ser Gln Ala Tyr Ala Arg Ser Gly Gly Ala Gly Asp Phe Glu 405 410 415	1352
AAG TGT CTT GCG ACG ACG GTG TGAGATGGGG TAATCTACGG TGAAGCAGCG Lys Cys Leu Ala Thr Thr Val 420	1403
GAGAGCCTCT CAACGAATGC AAAGGATAGG TTCGAGGCTT ACTTCATCAA CCTATATCAT	1463
CATAGGACAA GCCCCCAAT AGCCAGACTC GTCGTTGAC ATCGTGTATG AAAATAACCC	1523
ACCCACGCAC TCCGCTGCCA CTATTCGCGT GTATCGCATA CTAGGCGTTT TCGCCCAGTT	1583
GAACATGAGC CCATTCTGTC CCCAGTGAAA AAAAAAAA AAAAAATTCC TGCAGGCCGC	1642

## (2) INFORMATION FOR SEQ ID NO: 28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 442 amino acids

(B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Met His L u Gly Phe Val Thr Leu Ala Cys Leu Ile His Leu Ser Glu  
 -19                -15                -10                -5  
 Val Phe Ala Ala Ser Val Pro Arg Asn Ile Ala Pro Lys Phe Ser Ile  
 1                5                10  
 Pro Glu Ser Glu Gln Arg Asn Trp Ser Pro Tyr Ser Pro Tyr Phe Pro  
 15                20                25  
 Leu Ala Glu Tyr Lys Ala Pro Pro Ala Gly Cys Glu Ile Asn Gln Val  
 30                35                40                45  
 Asn Ile Ile Gln Arg His Gly Ala Arg Phe Pro Thr Ser Gly Ala Ala  
 50                55                60  
 Thr Arg Ile Lys Ala Gly Leu Ser Lys Leu Gln Ser Val Gln Asn Phe  
 65                70                75  
 Thr Asp Pro Lys Phe Asp Phe Ile Lys Ser Phe Thr Tyr Asp Leu Gly  
 80                85                90  
 Thr Ser Asp Leu Val Pro Phe Gly Ala Ala Gln Ser Phe Asp Ala Gly  
 95                100                105  
 Leu Glu Val Phe Ala Arg Tyr Ser Lys Leu Val Ser Ser Asp Asn Leu  
 110                115                120                125  
 Pro Phe Ile Arg Ser Asp Gly Ser Asp Arg Val Val Asp Thr Ala Thr  
 130                135                140  
 Asn Trp Thr Ala Gly Phe Ala Ser Ala Ser Arg Asn Ala Ile Gln Pro  
 145                150                155  
 Lys Leu Asp Leu Ile Leu Pro Gln Thr Gly Asn Asp Thr Leu Glu Asp  
 160                165                170  
 Asn Met Cys Pro Ala Ala Gly Glu Ser Asp Pro Gln Val Asp Ala Trp  
 175                180                185  
 Leu Ala Ser Ala Phe Pro Ser Val Thr Ala Gln Leu Asn Ala Ala Ala  
 190                195                200                205  
 Pro Gly Ala Asn Leu Thr Asp Ala Asp Ala Phe Asn Leu Val Ser Leu  
 210                215                220  
 Cys Pro Phe Met Thr Val Ser Lys Glu Gln Lys Ser Asp Phe Cys Thr  
 225                230                235  
 Leu Phe Glu Gly Ile Pro Gly Ser Phe Glu Ala Phe Ala Tyr Ala Gly  
 240                245                250  
 Asp Leu Asp Lys Phe Tyr Gly Thr Gly Tyr Gly Gln Ala Leu Gly Pro  
 255                260                265  
 Val Gln Gly Val Gly Tyr Ile Asn Glu Leu Leu Ala Arg Leu Thr Asn  
 270                275                280                285  
 Ser Ala Val Asn Asp Asn Thr Gln Thr Asn Arg Thr Leu Asp Ala Ala  
 290                295                300  
 Pro Asp Thr Phe Pro Leu Asn Lys Thr Met Tyr Ala Asp Phe Ser His  
 305                310                315  
 Asp Asn Leu Met Val Ala Val Phe Ser Ala Met Gly Leu Phe Arg Gln  
 320                325                330  
 Ser Ala Pro Leu Ser Thr Ser Thr Pro Asp Pro Asn Arg Thr Trp Leu  
 335                340                345  
 Thr Ser Ser Val Val Pro Phe Ser Ala Arg Met Ala Val Glu Arg Leu  
 350                355                360                365

Ser Cys Ala Gly Thr Thr Lys Val Arg Val Leu Val Gln Asp Gln Val  
 370 375 380  
 Gln Pro Leu Glu Phe Cys Gly Gly Asp Gln Asp Gly Leu Cys Ala Leu  
 385 390 395  
 Asp Lys Phe Val Glu Ser Gln Ala Tyr Ala Arg Ser Gly Gly Ala Gly  
 400 405 410  
 Asp Phe Glu Lys Cys Leu Ala Thr Thr Val  
 415 420

## (2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1536 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Trametes pubescens  
 (B) STRAIN: CBS 100232

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 79..1407

(ix) FEATURE:  
 (A) NAME/KEY: mat\_peptide  
 (B) LOCATION: 130..1407

(ix) FEATURE:  
 (A) NAME/KEY: sig\_peptide  
 (B) LOCATION: 79..129

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GGATCCGAAT TCGCCCCCAC ATTCGTTCCA TCTTAGCAGC CGTCCGCGCC CAGGTCTTCG	60
ATAACCCCCC GCGTGACT ATG GCC TTC TCA ATC TTG GCC TCG CTG CTC TTC Met Ala Phe Ser Ile Leu Ala Ser Leu Leu Phe -17 -15 -10	111
GTG TGT TAT GCA TAC GCC AGG GCT GTG CCC CGT GCA CAT ATC CCG CTC Val Cys Tyr Ala Tyr Ala Arg Ala Val Pro Arg Ala His Ile Pro Leu -5 1 5 10	159
CGC GAC ACC TCC GCG TGT CTA GAT GTA ACA CGC GAT GTG CAG CAG AGC Arg Asp Thr Ser Ala Cys Leu Asp Val Thr Arg Asp Val Gln Gln Ser 15 20 25	207
TGG TCC ATG TAC TCT CCC TAT TTC CCG GCA GCA ACT TAT GTG GCT CCG Trp Ser Met Tyr Ser Pro Tyr Phe Pro Ala Ala Thr Tyr Val Ala Pro 30 35 40	255
CCC GCG AGT TGC CAG ATC AAT CAG GTC CAC ATC ATC CAA CGT CAT GGT Pro Ala Ser Cys Gln Ile Asn Gln Val His Ile Ile Gln Arg His Gly 45 50 55	303
GCA CGC TTT CCC ACG TCT GGC GCA GCA AAG CGC ATC CAG ACA GCA GTA Ala Arg Phe Pro Thr Ser Gly Ala Ala Lys Arg Ile Gln Thr Ala Val 60 65 70	351
GCG AAG CTG AAG GCC GCG TCC AAC TAC ACC GAT CCC CTG CTC GCG TTC Ala Lys Leu Lys Ala Ala Ser Asn Tyr Thr Asp Pro Leu Leu Ala Phe 75 80 85 90	399
GTT ACG AAC TAC ACC TAC AGC TTA GGT CAG GAC AGC CTC GTT GAA CTC Val Thr Asn Tyr Thr Ser Leu Gly Gln Asp Ser Leu Val Glu Leu 95 100 105	447

GGT GCG ACT CAG TCC TCC GAA GCG GGC CAG GAG GCA TTC ACG CGG TAC Gly Ala Thr Gln Ser Ser Glu Ala Gly Gln Glu Ala Phe Thr Arg Tyr 110 115 120	495
TCA TCC CTC GTG AGC GCG GAC GAG CTT CCC TTC GTT CGG GCG TCG GGC Ser Ser Leu Val Ser Ala Asp Glu Leu Pro Phe Val Arg Ala Ser Gly 125 130 135	543
TCA GAT CGC GTC GTT GCG ACT GCC AAC AAC TGG ACT GCA GGT TTC GCG Ser Asp Arg Val Val Ala Thr Ala Asn Asn Trp Thr Ala Gly Phe Ala 140 145 150	591
CTT GCG AGC TCA AAC AGC ATC ACG CCC GTG CTC TCA GTC ATC ATT TCC Leu Ala Ser Ser Asn Ser Ile Thr Pro Val Leu Ser Val Ile Ile Ser 155 160 165 170	639
GAA GCG GGC AAT GAC ACC CTC GAC GAC AAC ATG TGC CCC GCT GCA GGC Glu Ala Gly Asn Asp Thr Leu Asp Asp Asn Met Cys Pro Ala Ala Gly 175 180 185	687
GAT TCG GAT CCC CAG GTC AAT CAA TGG CTC GCG CAG TTC GCA CCG CCG Asp Ser Asp Pro Gln Val Asn Gln Trp Leu Ala Gln Phe Ala Pro Pro 190 195 200	735
ATG ACT GCT CGC CTC AAC GCA GGC GCG CCC GGC GCG AAC CTC ACG GAC Met Thr Ala Arg Leu Asn Ala Gly Ala Pro Gly Ala Asn Leu Thr Asp 205 210 215	783
ACG GAC ACC TAC AAC CTG CTC ACG CTA TGC CCG TTC GAG ACT GTA GCC Thr Asp Thr Tyr Asn Leu Leu Thr Leu Cys Pro Phe Glu Thr Val Ala 220 225 230	831
ACC GAG CGG CGT AGT GAA TTC TGC GAC ATC TAC GAG GAG CTG CAG GCG Thr Glu Arg Arg Ser Glu Phe Cys Asp Ile Tyr Glu Glu Leu Gln Ala 235 240 245 250	879
GAA GAC GCC TTC GCG TAC AAT GCC GAT CTC GAC AAG TTC TAC GGC ACT Glu Asp Ala Phe Ala Tyr Asn Ala Asp Leu Asp Lys Phe Tyr Gly Thr 255 260 265	927
GGA TAC GGC CAG CCC CTC GGA CCC GTG CAA GGC GTC GGG TAC ATC AAC Gly Tyr Gly Gln Pro Leu Gly Pro Val Gln Gly Val Gly Tyr Ile Asn 270 275 280	975
GAG CTC ATC GCG CGC CTC ACC GCG CAG AAC GTG TCC GAC CAC ACG CAG Glu Leu Ile Ala Arg Leu Thr Ala Gln Asn Val Ser Asp His Thr Gln 285 290 295	1023
ACG AAC AGC ACA CTC GAC TCC TCG CCC GAG ACG TTC CCG CTC AAC CGC Thr Asn Ser Thr Leu Asp Ser Ser Pro Glu Thr Phe Pro Leu Asn Arg 300 305 310	1071
ACG CTC TAC GCG GAC TTC TCG CAC GAC AAC CAG ATG GTC GCG ATC TTC Thr Leu Tyr Ala Asp Phe Ser His Asp Asn Gln Met Val Ala Ile Phe 315 320 325 330	1119
TCG GCC ATG GGT CTC TTC AAC CAG TCC GCG CCG CTC GAC CCG ACG ACG Ser Ala Met Gly Leu Phe Asn Gln Ser Ala Pro Leu Asp Pro Thr Thr 335 340 345	1167
CCC GAC CCC GCG CGC ACG TTC CTC GTC AAG AAG ATC GTG CCG TTC TCC Pro Asp Pro Ala Arg Thr Phe Leu Val Lys Lys Ile Val Pro Phe Ser 350 355 360	1215
GCG CGC ATG GTC GTC GAG CGC CTC GAC TGC GGC GGT GCG CAG AGC GTG Ala Arg Met Val Val Glu Arg Leu Asp Cys Gly Gly Ala Gln Ser Val 365 370 375	1263
CGC CTG CTC GTG AAC GAC GCA GTG CAG CCG CTG GCG TTC TGC GGG GCG Arg Leu Leu Val Asn Asp Ala Val Gln Pro Leu Ala Phe Cys Gly Ala 380 385 390	1311
GAC ACG AGC GGG GTG TGC ACG CTG GAC GCG TTT GTC GAG AGC CAG GCG Asp Thr Ser Gly Val Cys Thr Leu Asp Ala Phe Val Glu Ser Gln Ala 395 400 405 410	1359

TAC GCG CGG AAC GAT GGC GAG GGC GAC TTC GAG AAG TGC TTC GCG ACA Tyr Ala Arg Asn Asp Gly Glu Gly Asp Phe Glu Lys Cys Phe Ala Thr 415 420 425	1407
TAGTTCCAGG TGTAGATACC CGGGGAAGAT GTACTCTCTA GACACCTCGC ATGTACTTAT CGATTAGAAA GAGACCCTGG CTGCTCTGCC CTCAAAAAAA AAAAAAAA AAAAAATTCC TGCGGCCCGC	1467 1527 1536

## (2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 443 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Met Ala Phe Ser Ile Leu Ala Ser Leu Leu Phe Val Cys Tyr Ala Tyr -17 -15 -10 -5
Ala Arg Ala Val Pro Arg Ala His Ile Pro Leu Arg Asp Thr Ser Ala 1 5 10 15
Cys Leu Asp Val Thr Arg Asp Val Gln Gln Ser Trp Ser Met Tyr Ser 20 25 30
Pro Tyr Phe Pro Ala Ala Thr Tyr Val Ala Pro Pro Ala Ser Cys Gln 35 40 45
Ile Asn Gln Val His Ile Ile Gln Arg His Gly Ala Arg Phe Pro Thr 50 55 60
Ser Gly Ala Ala Lys Arg Ile Gln Thr Ala Val Ala Lys Leu Lys Ala 65 70 75
Ala Ser Asn Tyr Thr Asp Pro Leu Leu Ala Phe Val Thr Asn Tyr Thr 80 85 90 95
Tyr Ser Leu Gly Gln Asp Ser Leu Val Glu Leu Gly Ala Thr Gln Ser 100 105 110
Ser Glu Ala Gly Gln Glu Ala Phe Thr Arg Tyr Ser Ser Leu Val Ser 115 120 125
Ala Asp Glu Leu Pro Phe Val Arg Ala Ser Gly Ser Asp Arg Val Val 130 135 140
Ala Thr Ala Asn Asn Trp Thr Ala Gly Phe Ala Leu Ala Ser Ser Asn 145 150 155
Ser Ile Thr Pro Val Leu Ser Val Ile Ile Ser Glu Ala Gly Asn Asp 160 165 170 175
Thr Leu Asp Asp Asn Met Cys Pro Ala Ala Gly Asp Ser Asp Pro Gln 180 185 190
Val Asn Gln Trp Leu Ala Gln Phe Ala Pro Pro Met Thr Ala Arg Leu 195 200 205
Asn Ala Gly Ala Pro Gly Ala Asn Leu Thr Asp Thr Asp Thr Tyr Asn 210 215 220
Leu Leu Thr Leu Cys Pro Phe Glu Thr Val Ala Thr Glu Arg Arg Ser 225 230 235
Glu Phe Cys Asp Ile Tyr Glu Glu Leu Gln Ala Glu Asp Ala Phe Ala 240 245 250 255
Tyr Asn Ala Asp Leu Asp Lys Phe Tyr Gly Thr Gly Tyr Gly Gln Pro 260 265 270
Leu Gly Pro Val Gln Gly Val Gly Tyr Ile Asn Glu Leu Ile Ala Arg 275 280 285

Leu Thr Ala Gln Asn Val Ser Asp His Thr Gln Thr Asn Ser Thr Leu  
 290 295 300  
 Asp Ser Ser Pro Glu Thr Phe Pro Leu Asn Arg Thr Leu Tyr Ala Asp  
 305 310 315  
 Phe Ser His Asp Asn Gln Met Val Ala Ile Phe Ser Ala Met Gly Leu  
 320 325 330 335  
 Phe Asn Gln Ser Ala Pro Leu Asp Pro Thr Thr Pro Asp Pro Ala Arg  
 340 345 350  
 Thr Phe Leu Val Lys Lys Ile Val Pro Phe Ser Ala Arg Met Val Val  
 355 360 365  
 Glu Arg Leu Asp Cys Gly Gly Ala Gln Ser Val Arg Leu Leu Val Asn  
 370 375 380  
 Asp Ala Val Gln Pro Leu Ala Phe Cys Gly Ala Asp Thr Ser Gly Val  
 385 390 395  
 Cys Thr Leu Asp Ala Phe Val Glu Ser Gln Ala Tyr Ala Arg Asn Asp  
 400 405 410 415  
 Gly Glu Gly Asp Phe Glu Lys Cys Phe Ala Thr  
 420 425

## (2) INFORMATION FOR SEQ ID NO: 31:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 276 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Schizophyllum sp.
  - (B) STRAIN: CBS 443.97
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (B) LOCATION: 1..276
  - (D) OTHER INFORMATION:/product= "PCR-fragment generated with SEQ ID NOS 15 and 17"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

TCTGCCGCAT CTGACGGTGT CTATAACCCC GTCCTCAACC TGATTATATC AGAAGAGCTT	60
AACGACACCC TCGATGATGC GATGTGCCCG AACGTCGGCG AATCGGACGC CCAAACGGAC	120
GAATGGACGT CTATTTACGC AGCCGCCATC GCTGAGCGTC TGAACAACAA CGCCGTGGGC	180
GCTAACCTGA CCACCACGAA CGTTTACAAC CTCATGTCTT TATGCCCTT CGACACGCTT	240
GCAGAAGGAGA CGCCGAGCCC CTTCTGCGAT CTCTTT	276

## (2) INFORMATION FOR SEQ ID NO: 32:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 92 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Schizophyllum sp.
  - (B) STRAIN: CBS 443.97

## (ix) FEATURE:

- (A) NAME/KEY: Region
- (B) LOCATION: 1..92
- (D) OTHER INFORMATION:/note= "the deduced amino acid sequence of SEQ ID NO: 31"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Ser Ala Ala Ser Asp Gly Val Tyr Asn Pro Val Leu Asn Leu Ile Ile  
1 5 10 15

Ser Glu Glu Leu Asn Asp Thr Leu Asp Asp Ala Met Cys Pro Asn Val  
20 25 30

Gly Glu Ser Asp Ala Gln Thr Asp Glu Trp Thr Ser Ile Tyr Ala Ala  
35 40 45

Pro Ile Ala Glu Arg Leu Asn Asn Asn Ala Val Gly Ala Asn Leu Thr  
50 55 60

Thr Thr Asn Val Tyr Asn Leu Met Ser Leu Cys Pro Phe Asp Thr Leu  
65 70 75 80

Ala Lys Glu Thr Pro Ser Pro Phe Cys Asp Leu Phe  
85 90

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 24, line 31 - 33.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an 

Name of depositary institution  
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (including postal code and country)

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit 4 December 1996

Accession Number Peniopora lycii  
CBS No. 686.96

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use  
only



This sheet was received with the international application

Authorized officer

Form PCT/RO/134 (July 1992)

For International Bureau use  
only



This sheet was received by the International Bureau on:

Authorized officer

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 24, line 35 - 37.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an 

Name of depositary institution  
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (including postal code and country)

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit 4 December 1996

Accession Number Agrocybe pediades  
CBS No. 900.96

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication or the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use  
only



This sheet was received with the international  
application

Authorized officer

Form PCT/RO/134 (July 1992)

For International Bureau use  
only



This sheet was received by the International Bureau  
on:

Authorized officer

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 25, line 1 - 3.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an 

Name of depositary institution  
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (including postal code and country)

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit 28 November 1997

Accession Number Paxillus involutus  
CBS No. 100231

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use  
only



This sheet was received with the international application

Authorized officer



Form PCT/RO/134 (July 1992)

For International Bureau use  
only



This sheet was received by the International Bureau on:

Authorized officer

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 25, line 5 - 8.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an 

Name of depositary institution  
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (including postal code and country)

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit 28 November 1997Accession Number Trametes pubescens  
CBS No. 100232

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use  
only



This sheet was received with the international application

Authorized officer



For International Bureau use  
only



This sheet was received by the International Bureau on:

Authorized officer

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 25, line 20 - 23.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an 

Name of depositary institution

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution (including postal code and country)

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit 2 December 1996

Accession Number Escherichia coli  
DSM No. 11312

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication of the mention or grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use  
only



This sheet was received with the international application

Authorized officer



For International Bureau use  
only



This sheet was received by the International Bureau on:

Authorized officer

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 25, line 24 - 26.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an 

Name of depositary institution  
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution (including postal code and country)

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit 2 December 1996

Accession Number Escherichia coli  
DSM No. 11313

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use

only

This sheet was received with the international application

Authorized officer



Form PCT/RO/134 (July 1992)

For International Bureau use

only

This sheet was received by the International Bureau on:

Authorized officer

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 25, line 28 - 30.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an 

Name of depositary institution  
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution (including postal code and country)

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit 12 November 1997Accession Number Escherichia coli  
DSM No. 11842

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use  
only



This sheet was received with the international application

Authorized officer



For International Bureau use  
only



This sheet was received by the International Bureau on:

Authorized officer

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 25, line 32 - 34.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an 

Name of depositary institution  
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution (including postal code and country)

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit 12 November 1997

Accession Number Escherichia coli  
DSM No. 11843

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet   
Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use —  
 only

This sheet was received with the international application

Authorized officer

Form

PCT/R0/134

For International Bureau use —  
 only

This sheet was received by the International Bureau on:

Authorized officer

(July 1992)

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 25, line 36 - 38.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an 

Name of depositary institution  
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution (including postal code and country)

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit 12 November 1997

Accession Number Escherichia coli  
DSM No. 11844

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use  
only



This sheet was received with the international application

Authorized officer

Form

PCT/R0/134

For International Bureau use  
only



This sheet was received by the International Bureau on:

Authorized officer

(July

1992)

## CLAIMS

1. An isolated polypeptide exhibiting phytase activity and being derived from the phylum Basidiomycota.

5

2. An isolated polypeptide exhibiting phytase activity and comprising at least one of the following amino acid sequences:

P-[Y/F]-[F/Y]-P-X-X-X-Y-X-X-P-P

10

(SEQ ID NO: 1)

Q-V-[N/H]-[I/L]-I-Q-R-H-G-A-R-[F/W]-P-T-S-G-A

(SEQ ID NO: 2)

15 I-Q-R-H-G-A-R-[F/W]-P-T-S-G-A-X-X-R

(SEQ ID NO: 3)

R-V-V-[D/A]-[S/T]-[A/S]-[T/N]-N-W-T-[A/E]-G-F

(SEQ ID NO: 4)

20

N-W-T-[A/E]-G-F-X-X-A-S

(SEQ ID NO: 5)

G-F-X-X-A-S-X-X-X-X-P

25

(SEQ ID NO: 6)

P-[N/D]-[P/E]-X-R-[T/L]-[W/F]-X-X-[S/K]-X-X-[V/T]-P-F-S

(SEQ ID NO: 7)

30 D-[Q/A]-[V/L]-Q-P-L-X-F-C-G-[G/A]

(SEQ ID NO: 8)

F-V-E-S-Q-X-[Y/F]-A-R-X-X-G-X-G-D-F-[E/A]-K-C

(SEQ ID NO: 9)

35

N-W-T-[A/E]-G-F

(SEQ ID NO: 10)

D-K-[F/Y]-Y-G-T

(SEQ ID NO: 11)

D-L-D-K-[F/Y]-Y-G

5

(SEQ ID NO: 12)

G-D-F-[A/E]-K

(SEQ ID NO: 13)

10 Q-V-[N/H]-[I/L]-I-Q

(SEQ ID NO: 14)

wherein "X" denotes any amino acid.

15 3. The polypeptide according to claim 2 comprising at least one of the following amino acid sequences, reference being had to the alignment at Fig. 7 and using the numbering corresponding to phyA\_P\_lycii:

(SEQ ID NO: 1) at position 46 to 57

20 (SEQ ID NO: 2) at position 64 to 80

(SEQ ID NO: 3) at position 68 to 83

(SEQ ID NO: 4) at position 155 to 167

(SEQ ID NO: 5) at position 162 to 171

(SEQ ID NO: 6) at position 166 to 177

25 (SEQ ID NO: 7) at position 358 to 373

(SEQ ID NO: 8) at position 395 to 405

(SEQ ID NO: 9) at position 415 to 433

(SEQ ID NO: 10) at position 162 to 167

(SEQ ID NO: 11) at position 273 to 278

30 (SEQ ID NO: 12) at position 271 to 277

(SEQ ID NO: 13) at position 428 to 432

(SEQ ID NO: 14) at position 64 to 69.

4. The polypeptide according to claim 3 comprising at least  
35 one of the following sets of amino acid sequences:

(SEQ ID NO: 10) and (SEQ ID NO: 12)

(SEQ ID NO: 10) and (SEQ ID NO: 13)

(SEQ ID NO: 14) and (SEQ ID NO: 10)

(SEQ ID NO: 14) and (SEQ ID NO: 12)  
(SEQ ID NO: 14) and (SEQ ID NO: 13)  
(SEQ ID NO: 11) and (SEQ ID NO: 13).

5 5. The polypeptide according to any of claims 1-4 comprising an amino acid sequence fulfilling one or more of the following conditions, reference being had to the alignment at Fig. 7 and using the numbering corresponding to phyA\_P\_lycii:

- (a) there are 10 amino acid residues between P46 and P57;
- 10 (b) there are 9 amino acid residues between F167 and P177;
- (c) there are 10 amino acid residues between P177 and N188;
- (d) there are 6 amino acid residues between C196 and D203;
- (e) there are 17 amino acid residues between L353 and P371.

15 6. An isolated polypeptide exhibiting phytase activity and being encoded by a DNA sequence, which hybridizes under conditions of medium to high stringency with the product of a PCR reaction using any suitable set of specific primers, which primers are derived from the alignment of fig. 6.

20

7. The polypeptide according to claim 6 wherein the set of primers consists of a sense primer and an anti-sense primer selected from amongst the following:

5' - CCC AAG CTT AAY TGG ACN GMN GGN TT - 3'

25

(SEQ ID NO: 15)

as a sense primer;

5' - CCC AAG CTT GAY AAR TWY GGN AC - 3'

30

(SEQ ID NO: 16)

as a sense primer;

5' - GCT CTA GAC RTA RWA YTT RTC NAR RTC - 3'

35

(SEQ ID NO: 17)

as an anti-sense primer;

144

5' - GCT CTA GAC AYT TNK CRA ART CNC C - 3'

as an anti-sense primer;

(SEQ ID NO: 18)

5

5' - CCC AAG CTT CAR GTN MAY MTN ATH CA - 3'

as a sense primer;

(SEQ ID NO: 19)

10

5' - GCT CTA GAC RAA NCC NKC NGT CCA RTT - 3'

as an anti-sense primer,

(SEQ ID NO: 20)

15 wherein N denotes any of A, C, G, T;  
R denotes any of A and G;  
Y denotes any of C and T;  
M denotes any of A and C; and  
W denotes any of A and T.

20

8. The polypeptide according to any of claims 6-7 wherein the set of primers is selected from amongst the following sets:

25

(SEQ ID NO: 15) and (SEQ ID NO: 17)  
(SEQ ID NO: 15) and (SEQ ID NO: 18)  
(SEQ ID NO: 19) and (SEQ ID NO: 20)  
(SEQ ID NO: 19) and (SEQ ID NO: 17)  
30 (SEQ ID NO: 19) and (SEQ ID NO: 18)  
(SEQ ID NO: 16) and (SEQ ID NO: 18).

9. An isolated polypeptide exhibiting phytase activity and  
35 which is a fungal 6-phytase.

10. The polypeptide according to any of claims 1-8 which is a (3+6)-phytase.

11. An isolated polypeptide exhibiting phytase activity and comprising the amino acid sequence of SEQ ID NO 22 or an amino acid sequence which is at least 50% homologous to this  
5 sequence.
12. An isolated polypeptide exhibiting phytase activity and comprising the amino acid sequence of amino acid no. 25, 27, 28 or 31 to 453 of SEQ ID NO 22 or an amino acid sequence  
10 which is at least 50% homologous to either of these sequences.
13. An isolated polypeptide exhibiting phytase activity and encoded by a phytase encoding part of
  - i) SEQ ID NO 21, or
  - 15 ii) the DNA sequence cloned into plasmid pYES 2.0 present in *Escherichia coli* DSM 11313, or an analogue or derivative thereof which is at least 50% homologous with said polypeptide.
- 20 14. An isolated polypeptide exhibiting phytase activity and comprising the amino acid sequence of SEQ ID NO 24 or an amino acid sequence which is at least 50% homologous to this sequence.
- 25 15. An isolated polypeptide exhibiting phytase activity and comprising the amino acid sequence of amino acid no. 31 to 439 of SEQ ID NO 24 or an amino acid sequence which is at least 50% homologous to this sequence.
- 30 16. An isolated polypeptide exhibiting phytase activity and encoded by a phytase encoding part of
  - i) SEQ ID NO 23, or
  - ii) the DNA sequence cloned into plasmid pYES 2.0 pre-sent in *Escherichia coli* DSM 11312, or
- 35 an analogue or derivative which is at least 50% homologous to said polypeptide.
17. An isolated polypeptide exhibiting phytase activity and comprising the amino acid sequence of SEQ ID NO 26 or an amino

acid sequence which is at least 50% homologous to this sequence.

18. An isolated polypeptide exhibiting phytase activity and  
5 encoded by a phytase encoding part of

i) SEQ ID NO 25, or

ii) the DNA sequence cloned into plasmid pYES 2.0 pre-sent in  
Escherichia coli 11842

or an analogue or derivative which is at least 50% homologous  
10 to said polypeptide.

19. An isolated polypeptide exhibiting phytase activity and  
comprising the amino acid sequence of SEQ ID NO 28 or an amino  
acid sequence which is at least 50% homologous to this  
15 sequence.

20. An isolated polypeptide exhibiting phytase activity and  
encoded by a phytase encoding part of

i) SEQ ID NO 27, or

20 ii) the DNA sequence cloned into plasmid pYES 2.0 pre-sent in  
Escherichia coli DSM 11843, or

an analogue or derivative which is at least 50% homologous to  
said polypeptide.

25 21. An isolated polypeptide exhibiting phytase activity and  
comprising the amino acid sequence of SEQ ID NO 30 or an amino  
acid sequence which is at least 50% homologous to this  
sequence.

30 22. An isolated polypeptide exhibiting phytase activity and  
encoded by a phytase encoding part of

i) SEQ ID NO 29, or

ii) the DNA sequence cloned into plasmid pYES 2.0 pre-sent in  
Escherichia coli DSM 11844, or

35 an analogue or derivative which is at least 50% homologous to  
said polypeptide.

23. A DNA molecule encoding the polypeptide according to any  
of claims 1-22.

24. A DNA molecule encoding a polypeptide exhibiting phytase activity and comprising at least one of the following DNA sequences:

5 5' - CCC AAG CTT AAY TGG ACN GMN GGN TT - 3'

(SEQ ID NO: 15)

5' - CCC AAG CTT GAY AAR TWY GGN AC - 3'

(SEQ ID NO: 16)

5' - GCT CTA GAC RTA RWA YTT RTC NAR RTC - 3'

10

(SEQ ID NO: 17)

5' - GCT CTA GAC AYT TNK CRA ART CNC C - 3'

(SEQ ID NO: 18)

5' - CCC AAG CTT CAR GTN MAY MTN ATH CA - 3'

(SEQ ID NO: 19)

15 5' - GCT CTA GAC RAA NCC NKC NGT CCA RTT - 3'

(SEQ ID NO: 20)

wherein N denotes any of A, C, G, T;

R denotes any of A and G;

Y denotes any of C and T;

20 M denotes any of A and C; and

W denotes any of A and T.

25 25. A DNA molecule encoding a polypeptide exhibiting phytase activity, and having a DNA sequence selected from the following

(a) a phytase encoding part of any of SEQ ID NOS: 21, 23, 25, 27 or 29;

30 (b) a phytase encoding part of any of the DNA sequences cloned into plasmid pYES 2.0 present in any of Escherichia coli DSM 11313, 11312, 11842, 11843, or 11844;

(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 55% homologous to said DNA sequence;

35 (d) a DNA sequence which is capable of hybridizing with the sequences of (a) or (b) under conditions of low stringency;

(e) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with the sequence of (a)

or (b), but which encodes a polypeptide comprising the amino acid sequence shown in any of SEQ ID NOS. 22, 24, 26, 28 or 30 or a fragment thereof.

5 26. A DNA molecule encoding a polypeptide exhibiting phytase activity, which DNA molecule hybridizes under conditions of medium/high stringency with the product of a PCR reaction using any suitable set of specific primers derived from the alignment of Fig. 6, or the primers mentioned in claim 7 or 8.

10

27. A specific primer suitable for use in a PCR reaction and derivable from the alignment of fig. 6.

28. The primer according to claim 27 which is selected from  
15 amongst the following:

5' - CCC AAG CTT AAY TGG ACN GMN GGN TT - 3'

(SEQ ID NO: 15)

5' - CCC AAG CTT GAY AAR TWY GGN AC - 3'

(SEQ ID NO: 16)

20 5' - GCT CTA GAC RTA RWA YTT RTC NAR RTC - 3'

(SEQ ID NO: 17)

5' - GCT CTA GAC AYT TNK CRA ART CNC C - 3'

(SEQ ID NO: 18)

5' - CCC AAG CTT CAR GTN MAY MTN ATH CA - 3'

(SEQ ID NO: 19)

25 5' - GCT CTA GAC RAA NCC NKC NGT CCA RTT - 3'

(SEQ ID NO: 20)

wherein N denotes any of A, C, G, T;

R denotes any of A and G;

30 Y denotes any of C and T;

M denotes any of A and C; and

W denotes any of A and T.

29. A vector comprising the DNA molecule according to any of  
35 claims 23-26.

30. A host cell comprising the DNA molecule according to any of claims 23-26 or the vector according to claim 29.

31. A method of identifying a phytase producing cell comprising the following steps:

- i) selecting a cell to provide a template;
- 5 ii) selecting, on the basis of the alignment of fig. 6, a set of specific primers suitable for PCR;
- iii) conducting a PCR reaction on this template using said primers to obtain an amplified PCR fragment derived from said template;
- 10 iv) verifying that the PCR fragment is specific; and
- v) identifying the cell having provided the template as a phytase producer.

15 32. The method according to claim 31, wherein the set of primers consists of a sense primer and an anti-sense primer selected from amongst the following:

5' - CCC AAG CTT AAY TGG ACN GMN GGN TT - 3'

(SEQ ID NO: 15)

20 as a sense primer;

5' - CCC AAG CTT GAY AAR TWY GGN AC - 3'

(SEQ ID NO: 16)

25 as a sense primer;

5' - GCT CTA GAC RTA RWA YTT RTC NAR RTC - 3'

(SEQ ID NO: 17)

30 as an anti-sense primer;

5' - GCT CTA GAC AYT TNK CRA ART CNC C - 3'

(SEQ ID NO: 18)

35 as an anti-sense primer;

5' - CCC AAG CTT CAR GTN MAY MTN ATH CA - 3'

(SEQ ID NO: 19)

as a sense primer;

5 5' - GCT CTA GAC RAA NCC NKC NGT CCA RTT - 3'

(SEQ ID NO: 20)

as an anti-sense primer,

wherein N denotes any of A, C, G, T;

10 R denotes any of A and G;

Y denotes any of C and T;

M denotes any of A and C; and

W denotes any of A and T.

15 33. The method according to claim 32, wherein the set of primers is selected from amongst the following sets:

(SEQ ID NO: 15) and (SEQ ID NO: 17)

(SEQ ID NO: 15) and (SEQ ID NO: 18)

20 (SEQ ID NO: 19) and (SEQ ID NO: 20)

(SEQ ID NO: 19) and (SEQ ID NO: 17)

(SEQ ID NO: 19) and (SEQ ID NO: 18)

(SEQ ID NO: 16) and (SEQ ID NO: 18). .

25 34. A process for preparing a polypeptide exhibiting phytase activity, the process comprising the following steps:

a) identifying a phytase producing cell using the method of any of claims 31-33; or

b) conducting step a) and further cloning a phytase encoding

30 DNA molecule from said phytase producing cell and transforming a host cell with said DNA molecule; or

c) using the amplified PCR fragment obtained according to the method of any of claims 31-33 as a hybridization probe to isolate a DNA molecule encoding a phytase

35 polypeptide, transforming a host cell with said DNA molecule;

and

d) culturing the cell obtained under a), b) or c) above under conditions permitting the production of the polypeptide,

and recovering the polypeptide from the culture broth.

35. A process for preparing a polypeptide exhibiting phytase activity, the process comprising culturing the host cell  
5 according to claim 30 under conditions permitting the production of the polypeptide, and recovering the polypeptide from the culture broth.

36. A feed or food comprising at least one polypeptide of any  
10 of claims 1-22 or at least one polypeptide obtainable according to any of claims 33-34.

37. A process for preparing a feed or food according to claim 36, wherein the at least one polypeptide is added to the food  
15 or feed components.

38. A composition comprising at least one polypeptide of any of claims 1-22; or at least one polypeptide obtainable according to any of claims 33-34.

20

39. The composition according to claim 38 suitable for use in food or feed preparations.

40. The composition according to any of claims 38-39 which is  
25 an animal feed additive.

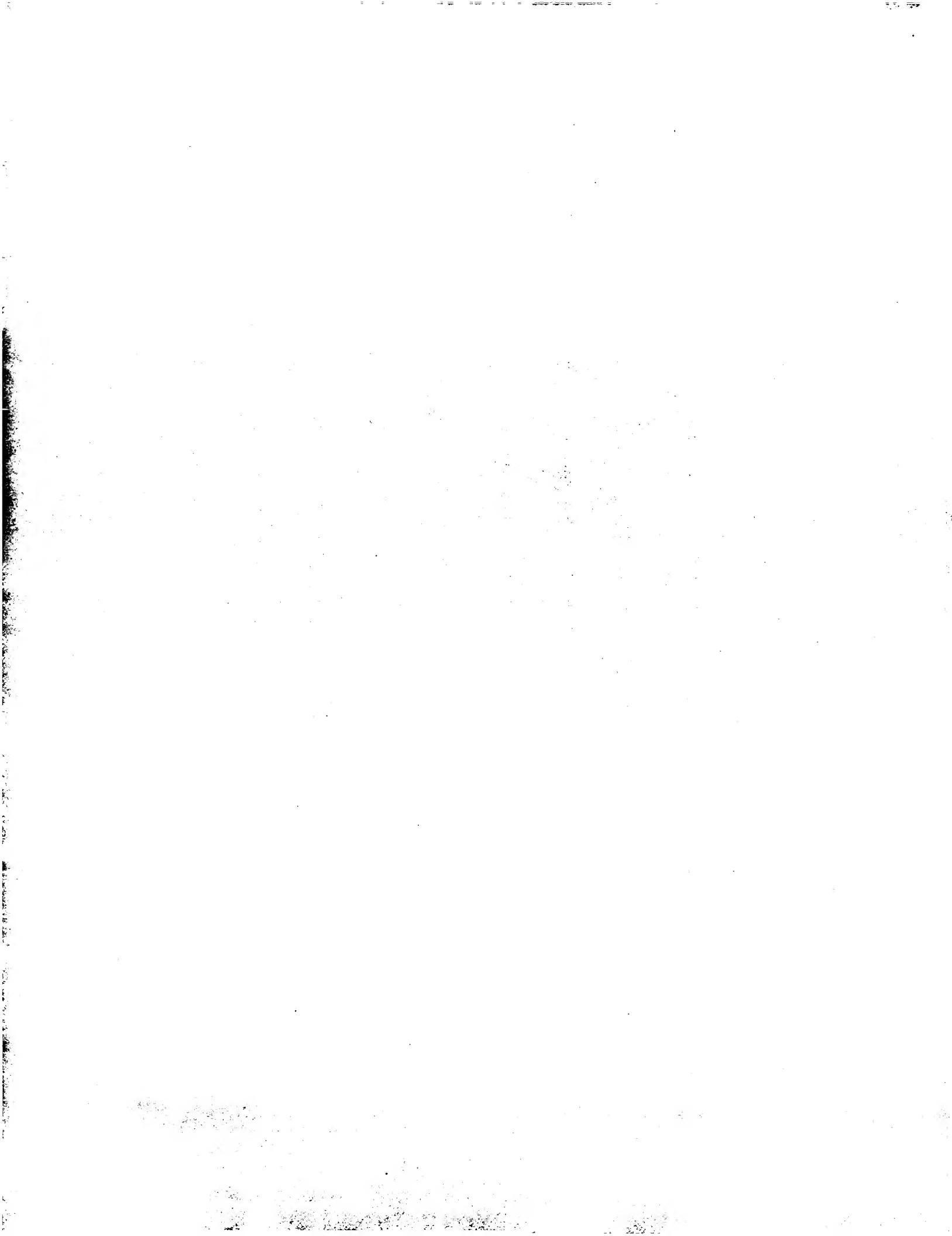
41. A process for reducing phytate levels in animal manure comprising feeding an animal with an effective amount of the feed according to claim 36 or obtainable according to  
30 claim 37.

42. Use of the polypeptide of any of claims 1-22; or the polypeptide obtainable according to any of claims 34-35; or the composition of any of claims 38-39 for liberating  
35 phosphorous from a phytase substrate.

43. Use of the polypeptide of any of claims 1-22; or the polypeptide obtainable according to any of claims 34-35; or the composition of any of claims 38-39 for improving the  
5 food or feed utilization.

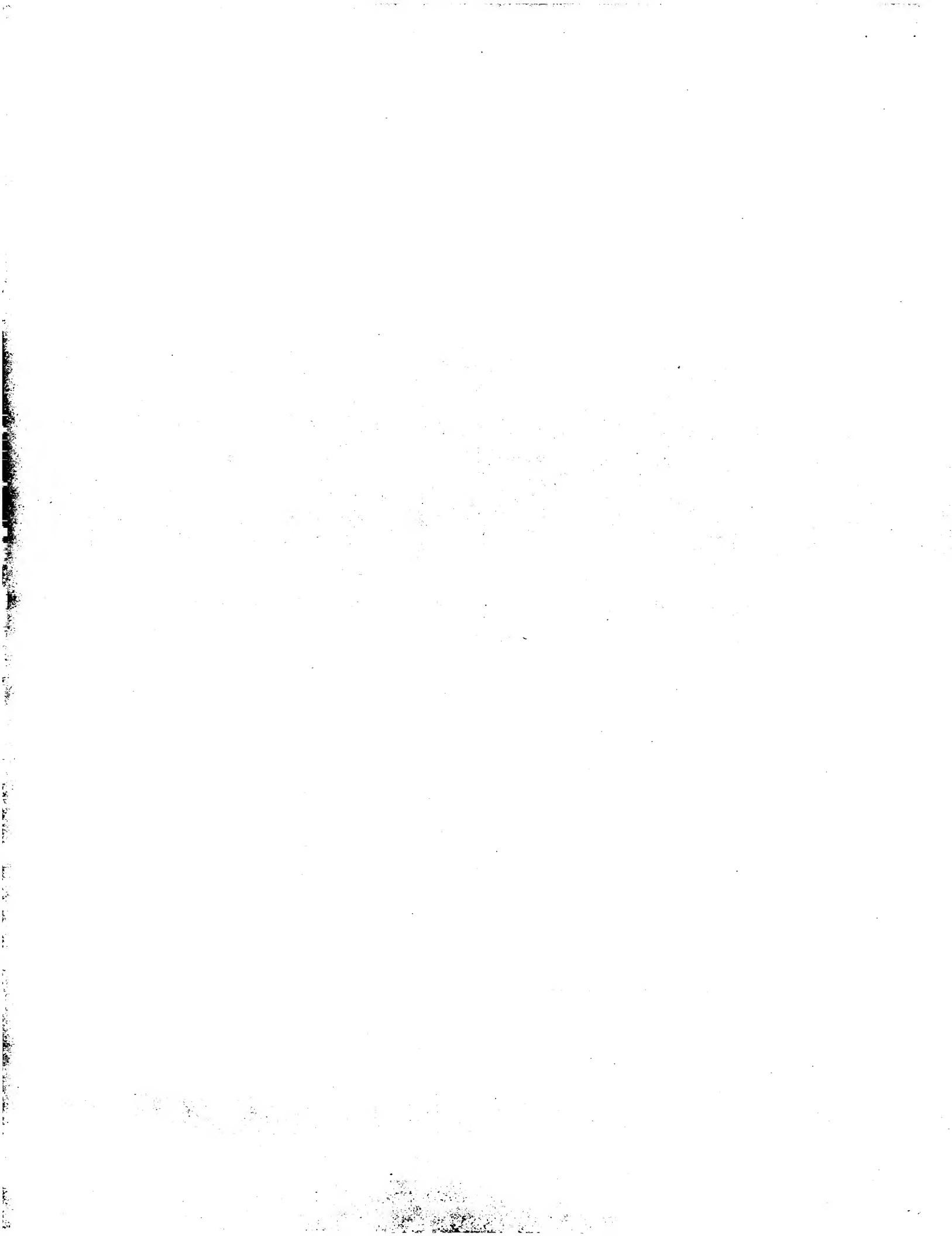
1/38

Fig. 1



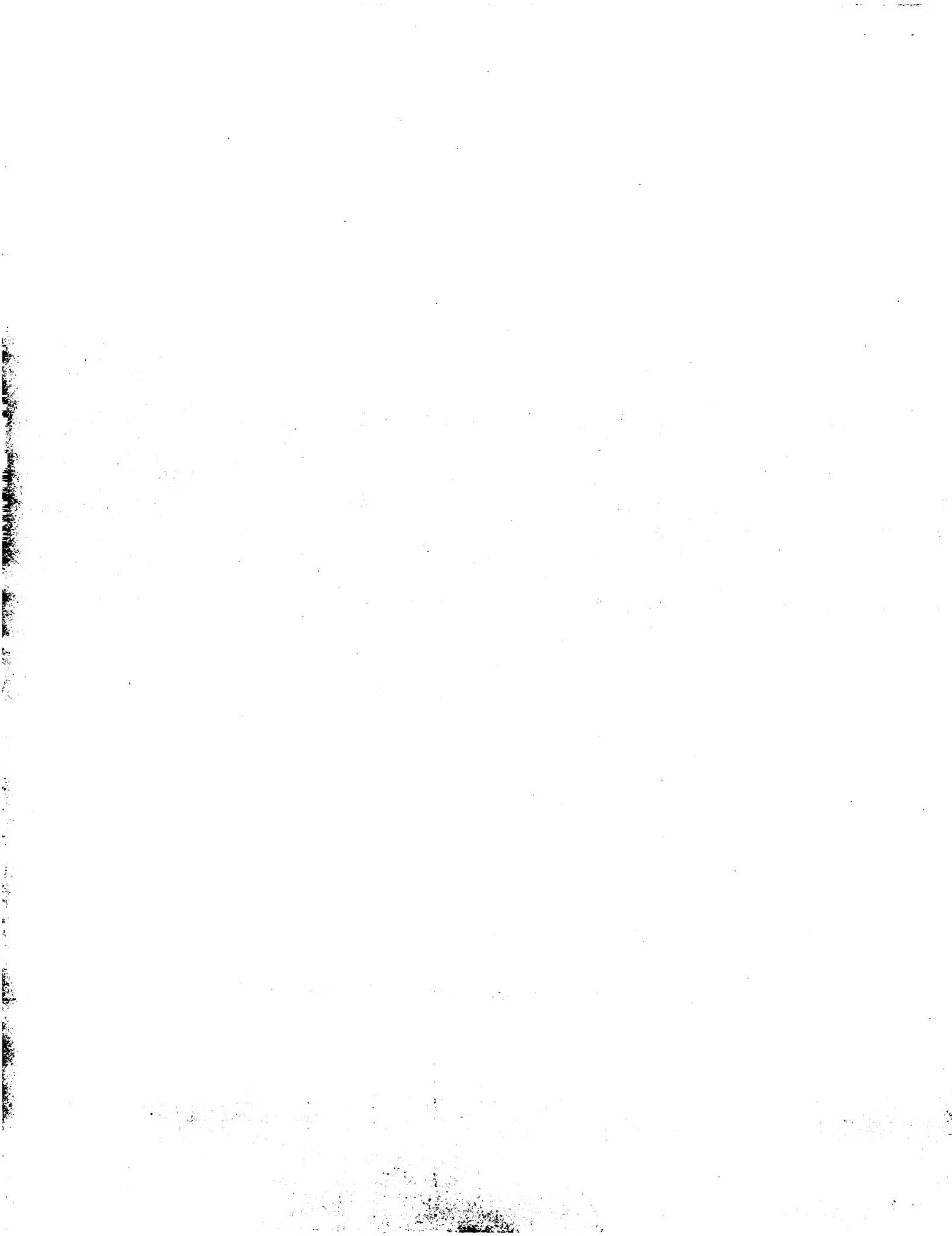
2/38

Fig. 2



3/38

Fig. 3



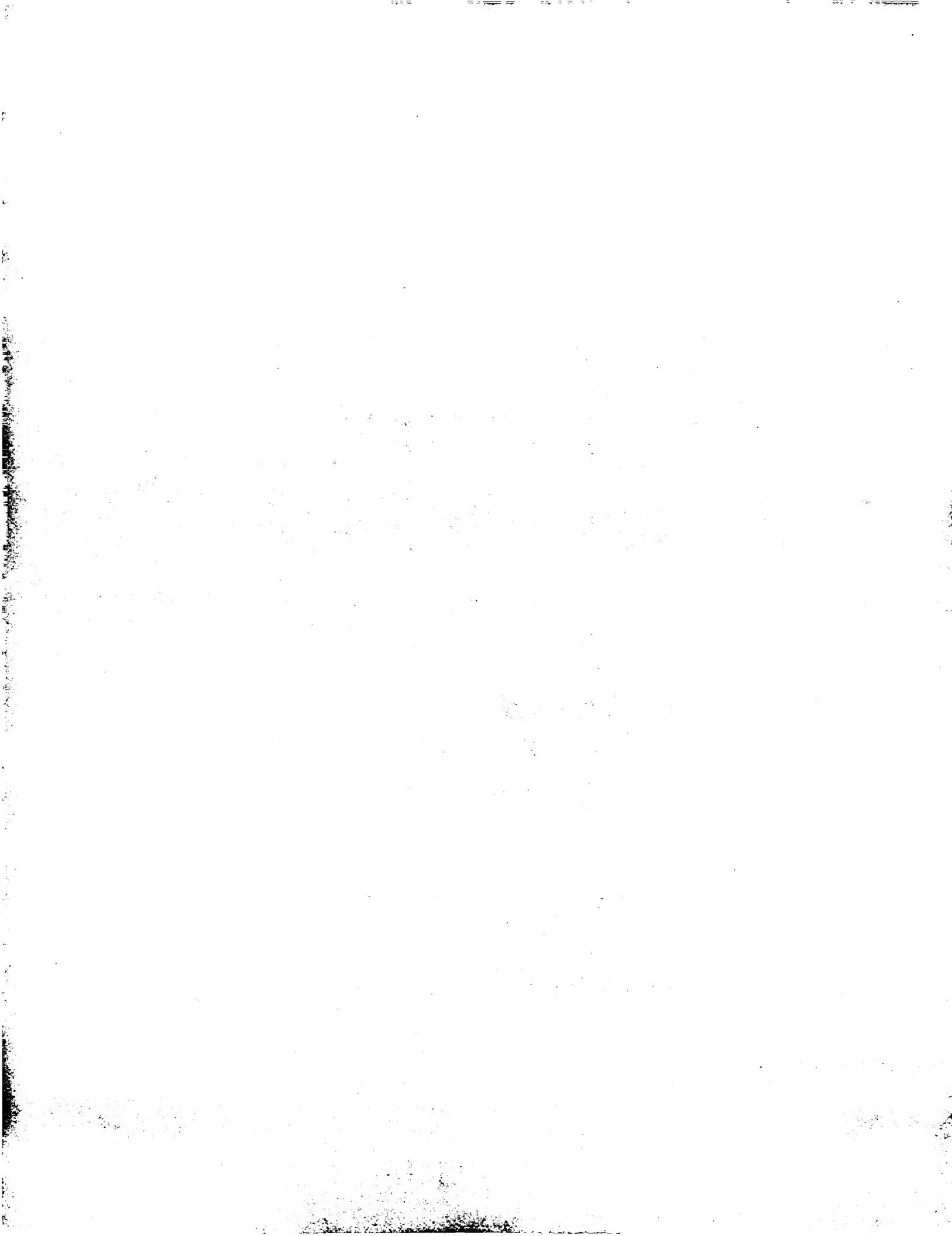
4/38

Fig. 4



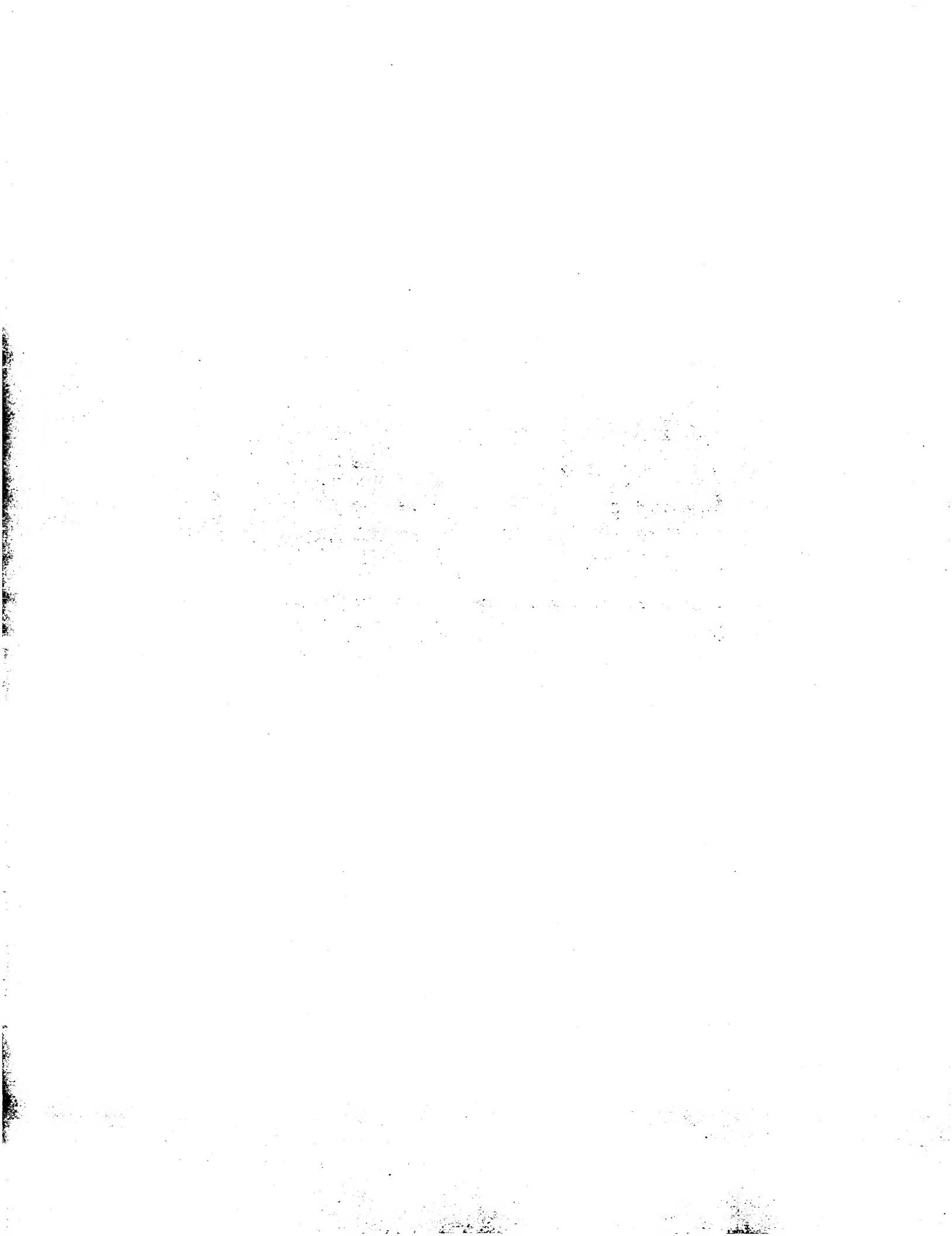
5/38

Fig. 5



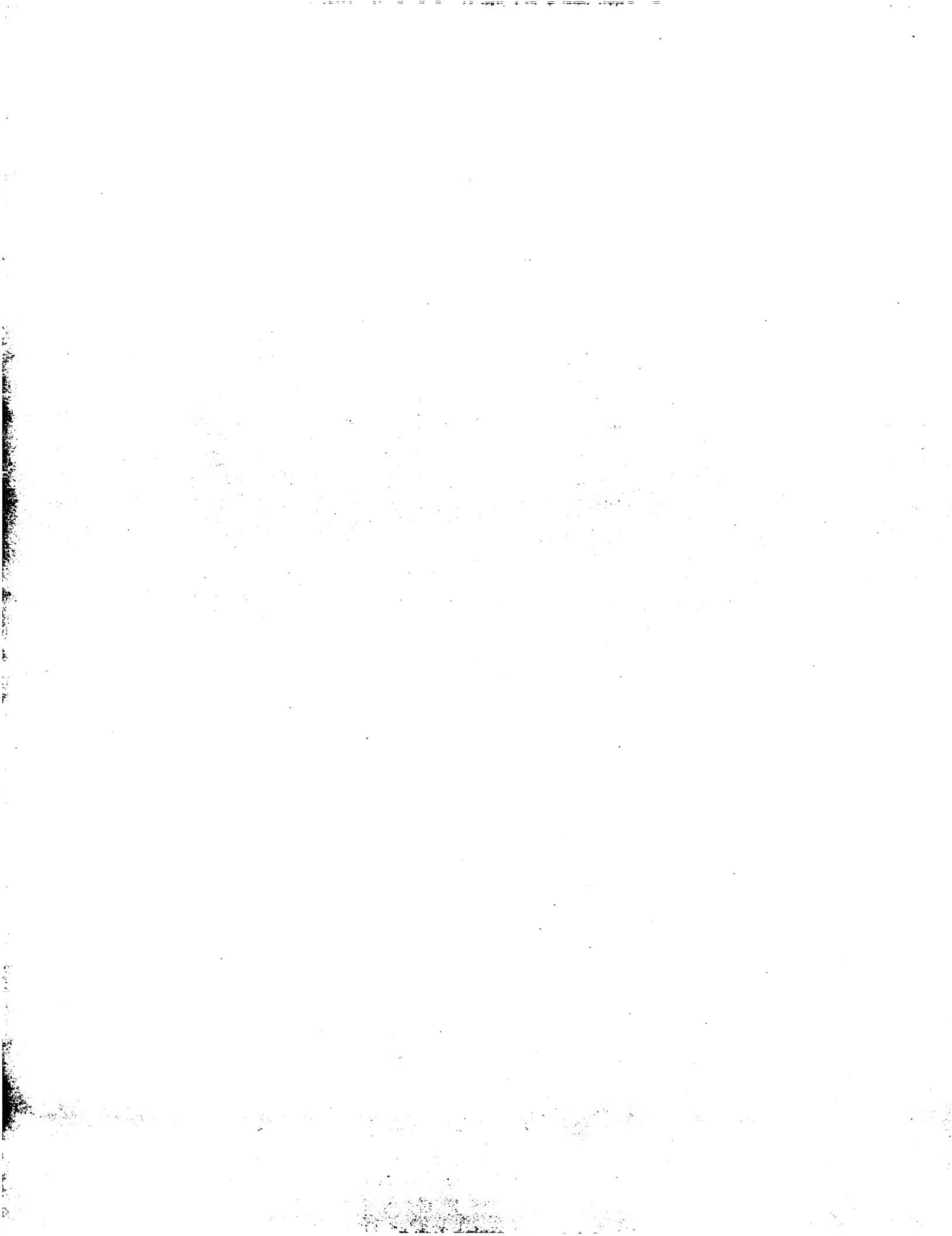
6/38

Fig. 6



7/38

Fig. 7



8/38

**Peniophora phytase pH-profile, 5mM phytate, 30 min at  
37°C**

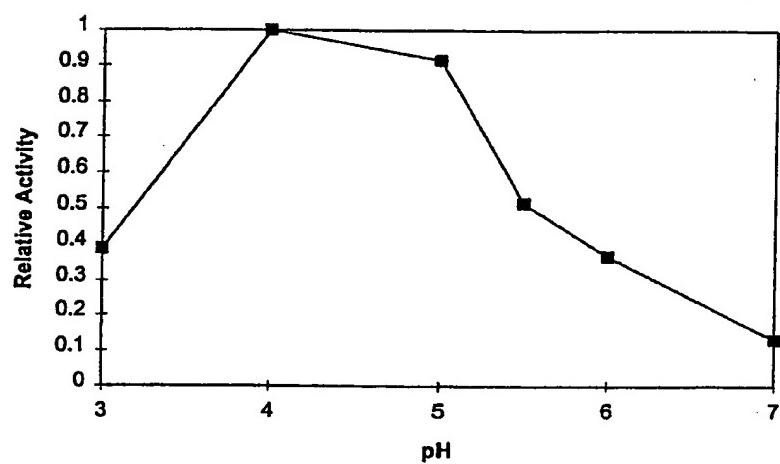
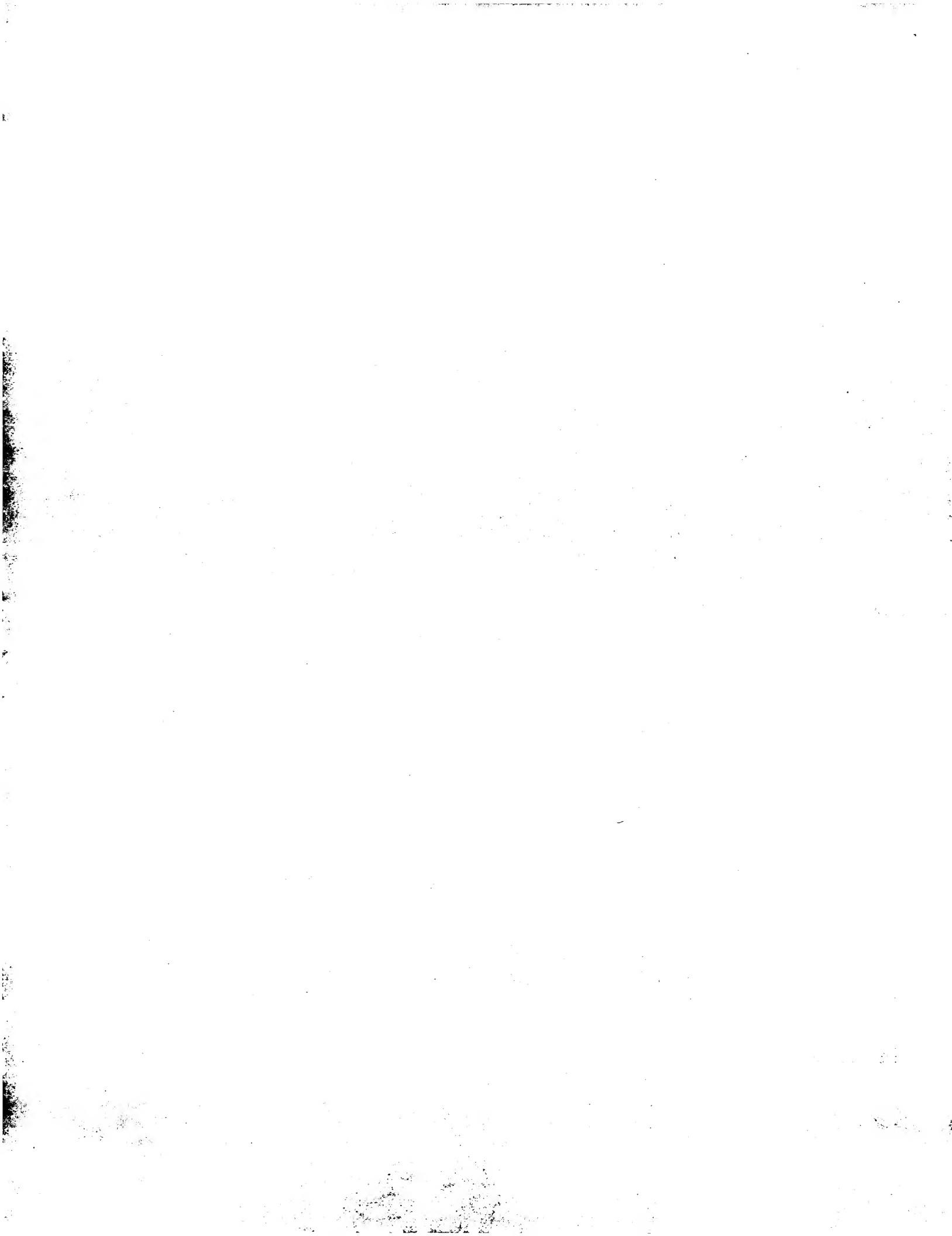


Fig. 8



9/38

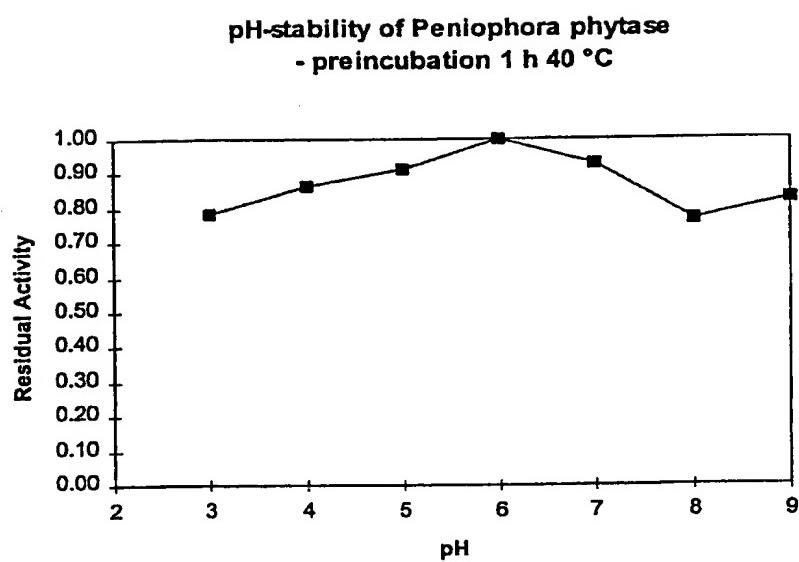
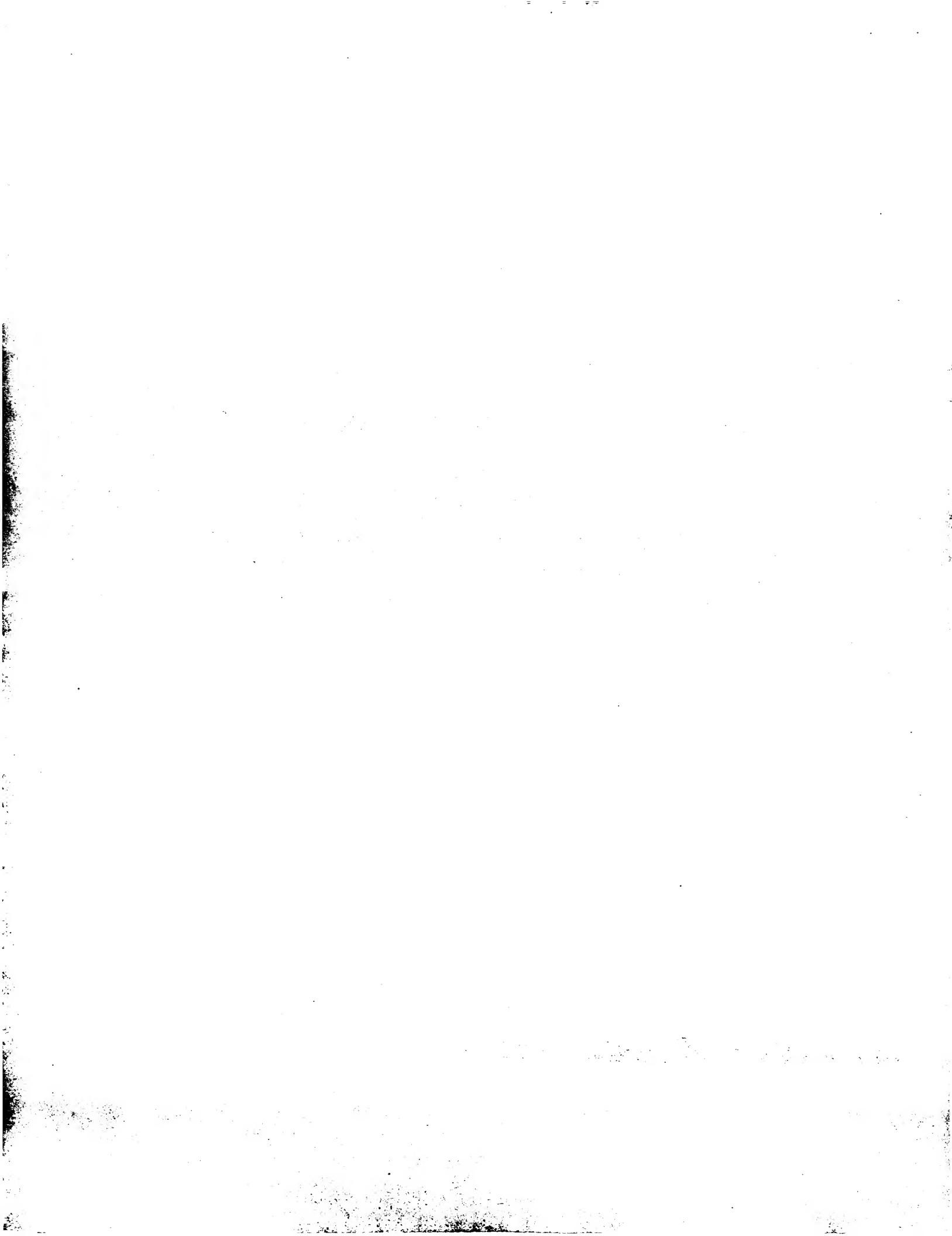


Fig. 9



10/38

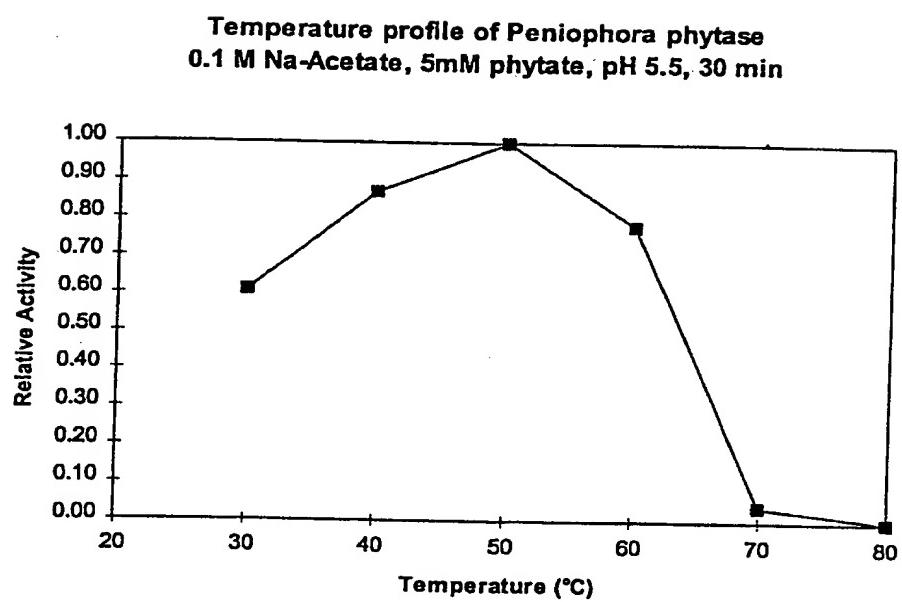
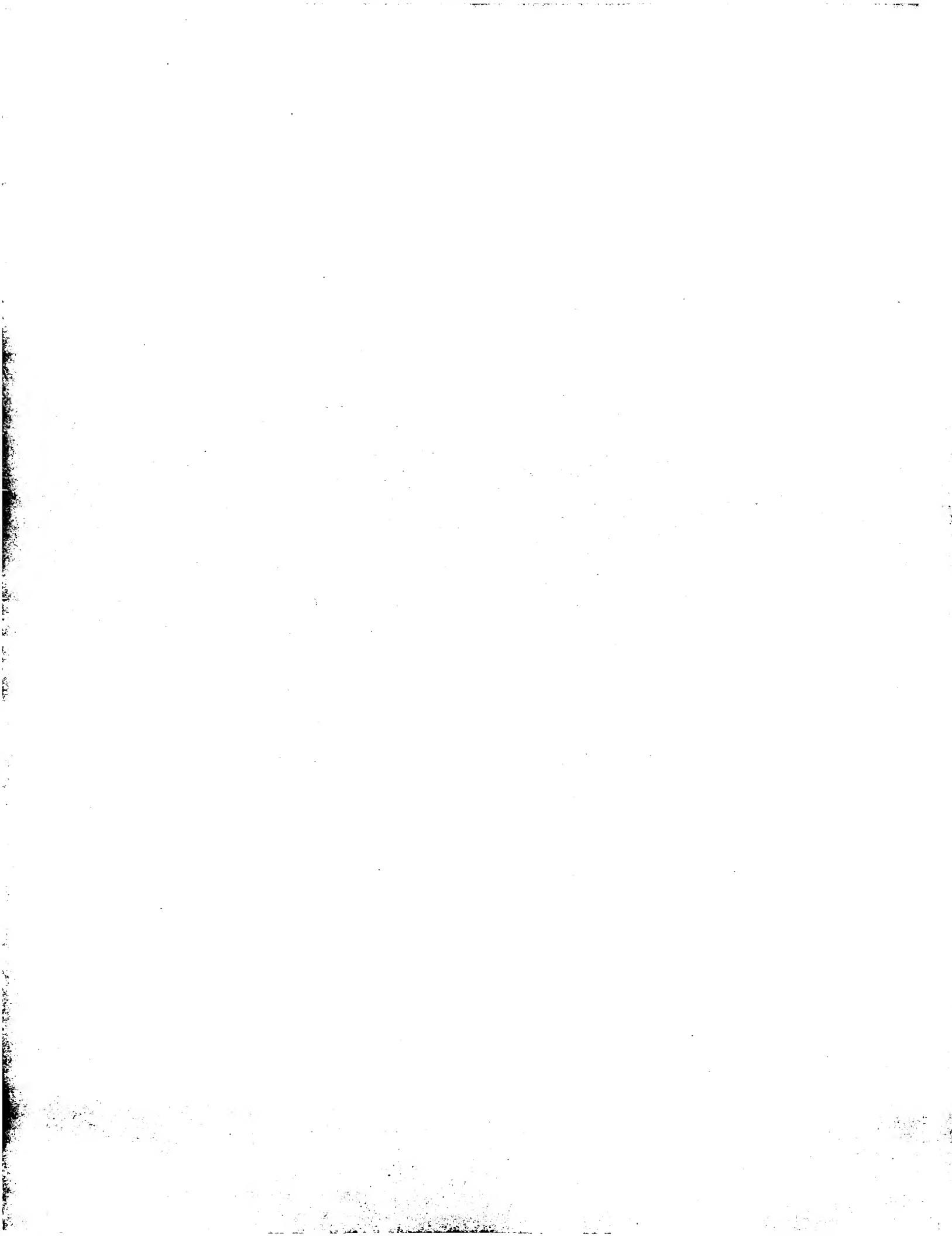
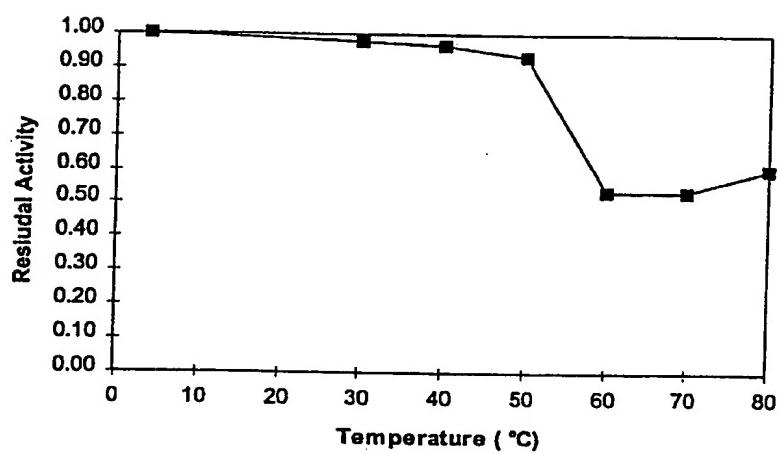


Fig. 10

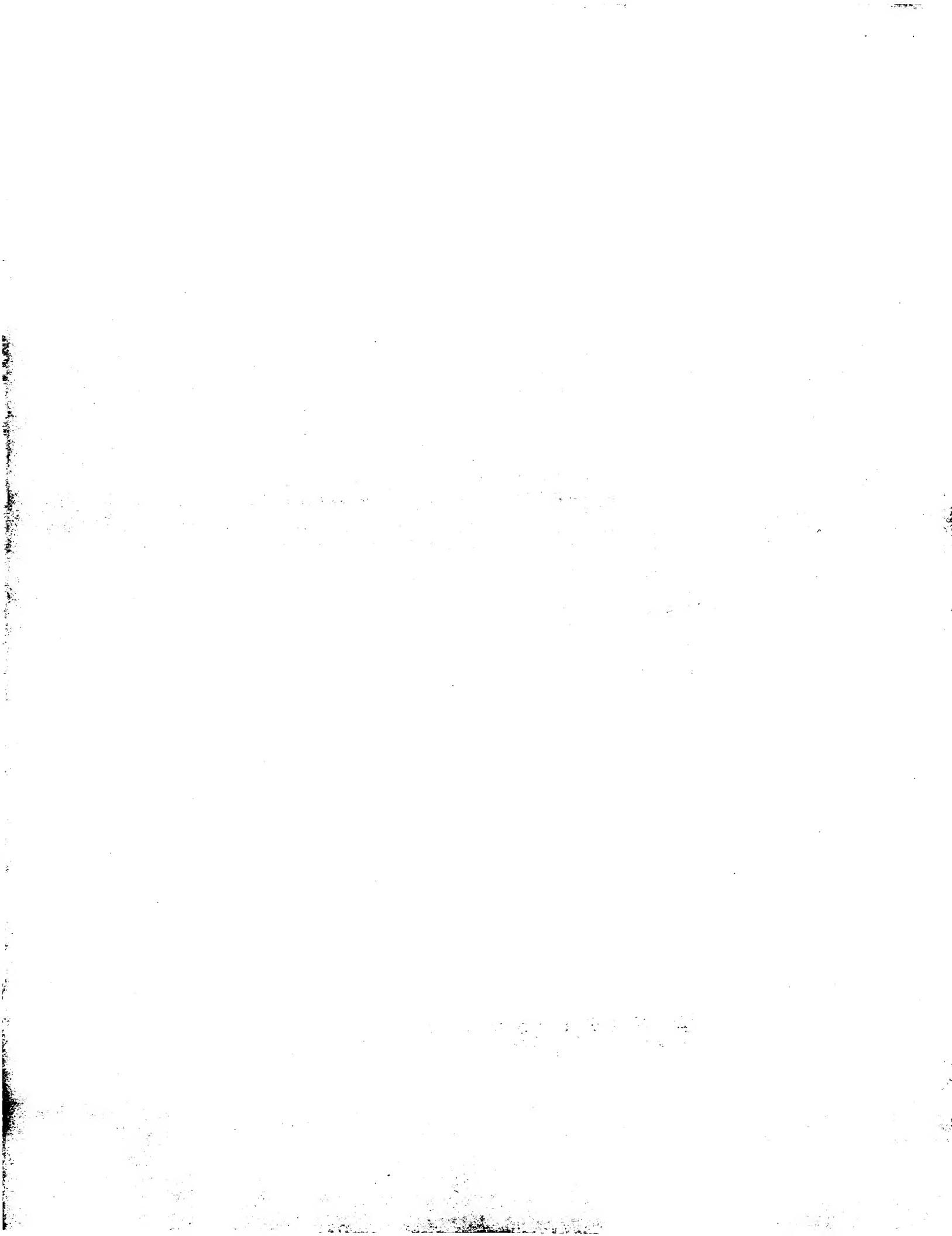


11/38

**Temperature stability of Peniohora phytase -  
preincubation 60 min in 0.1 M Na-acetate pH 5.5**



**Fig. 11**



12/38

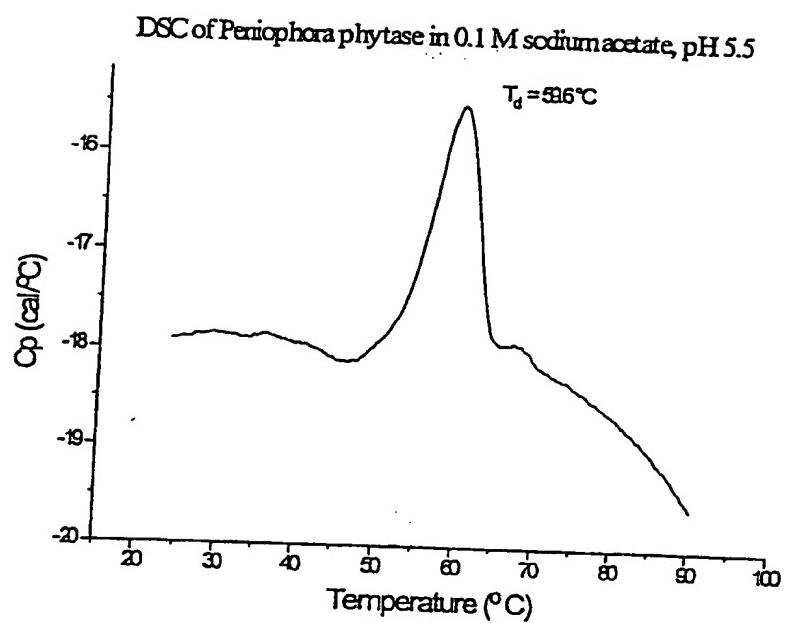
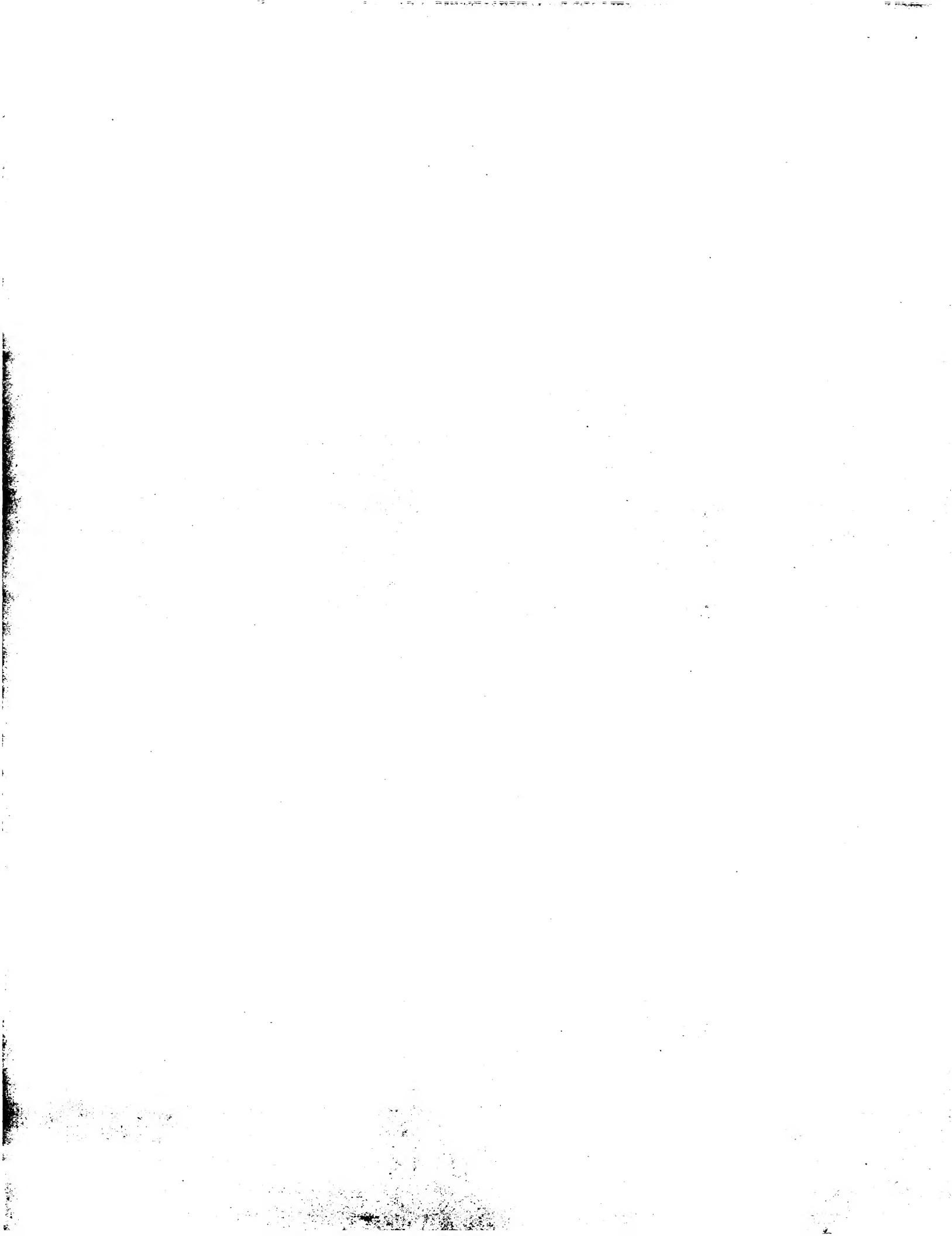


Fig. 12



13/38

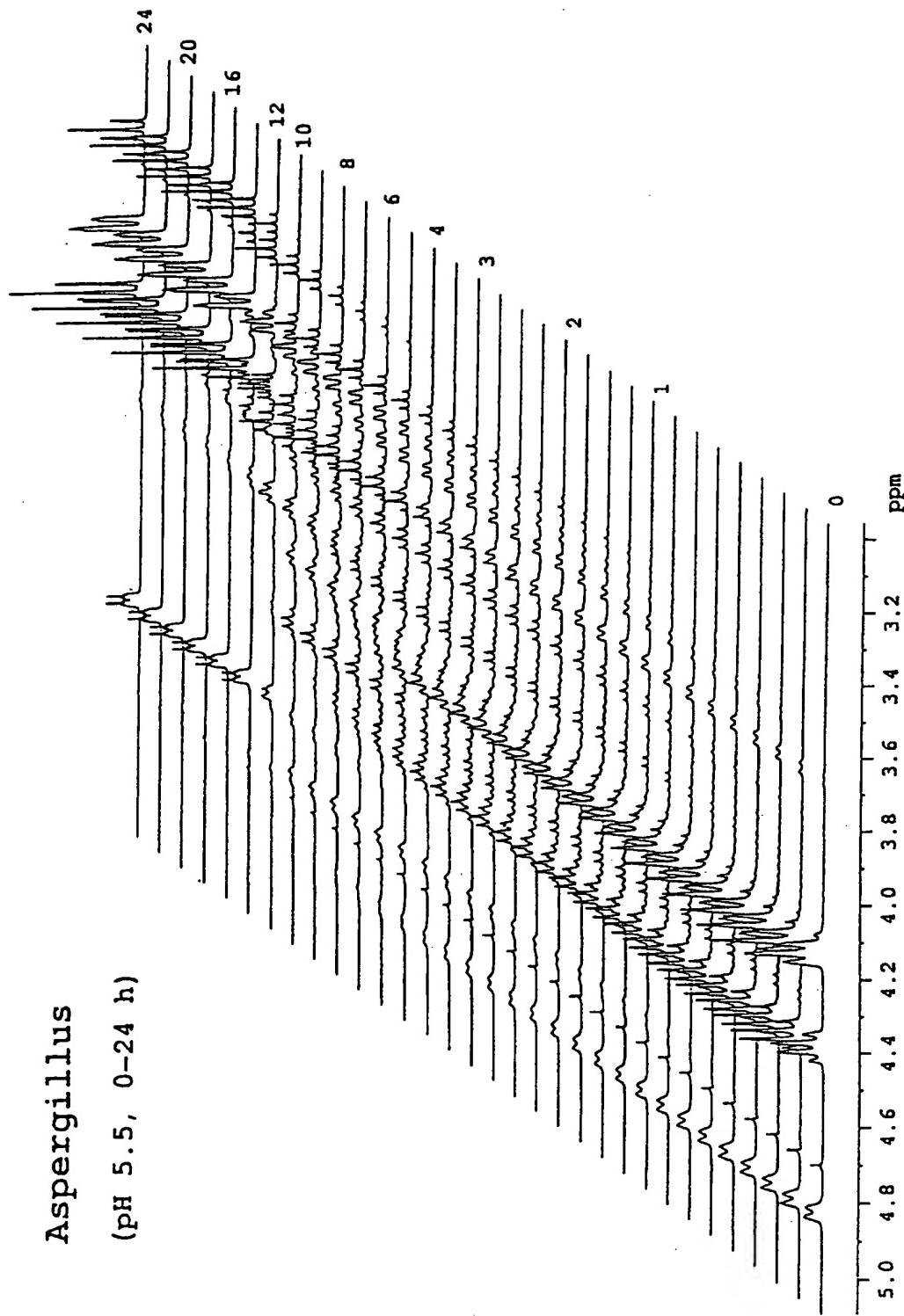
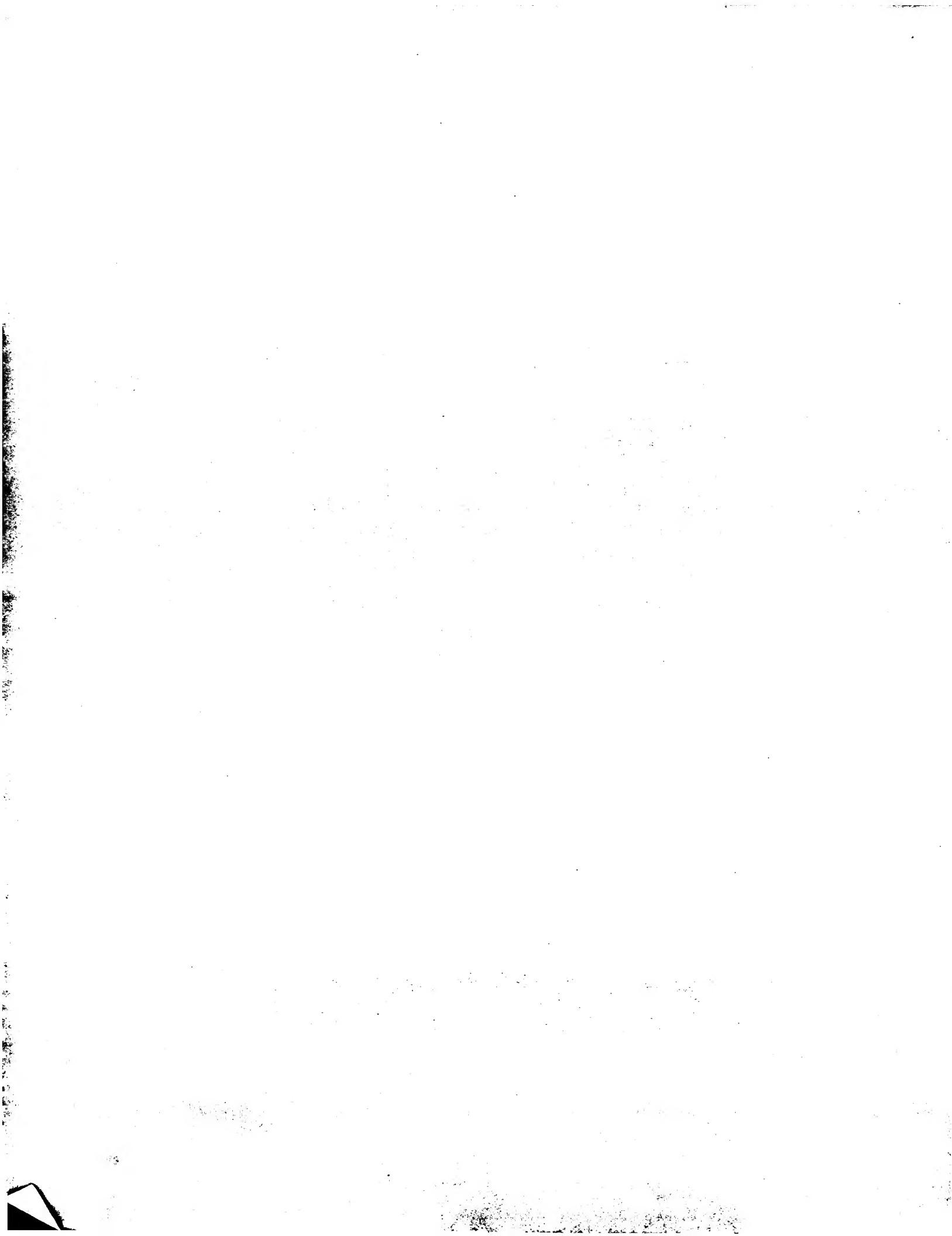


Fig. 13



14/38

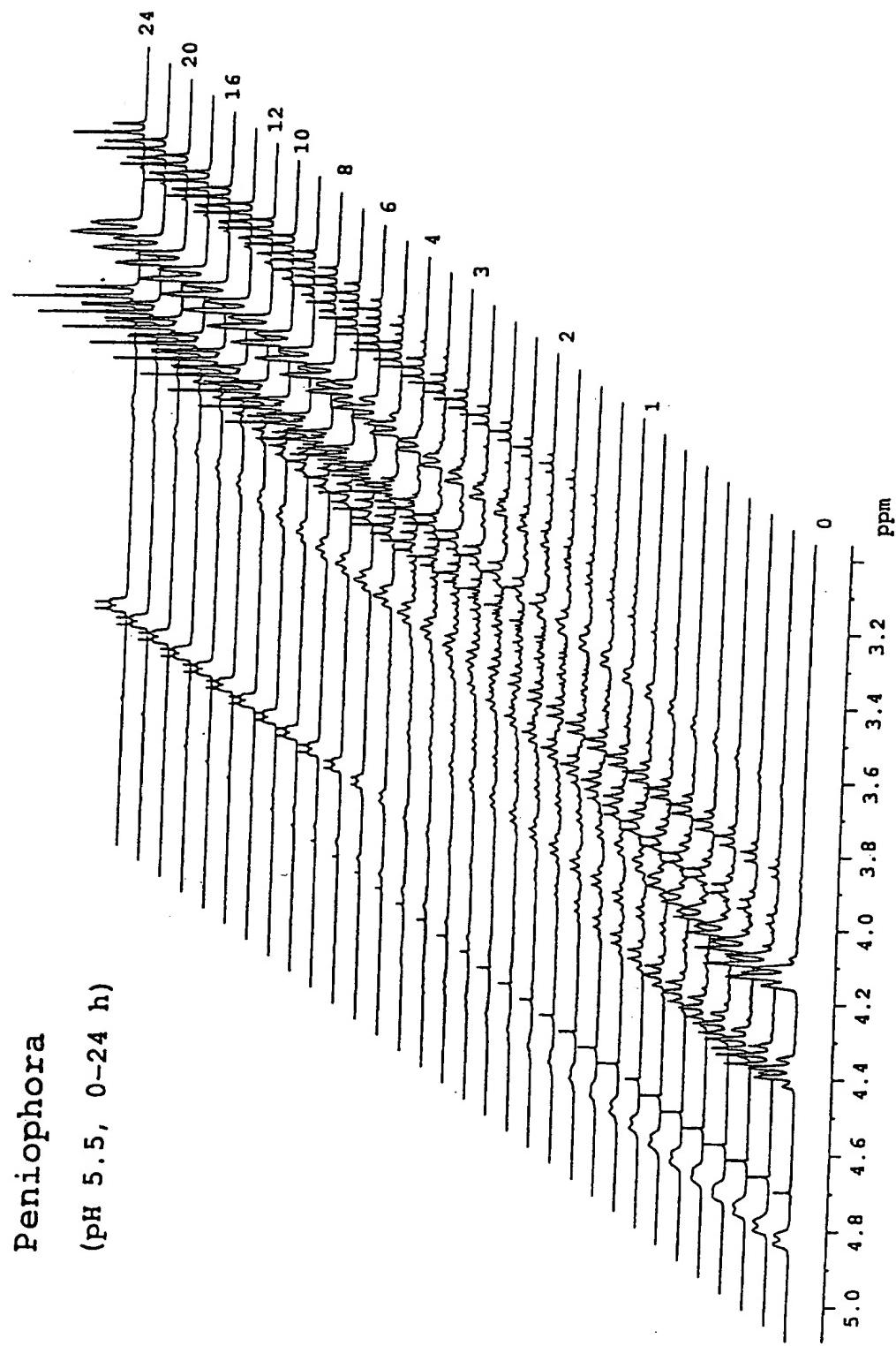


Fig. 14



15/38

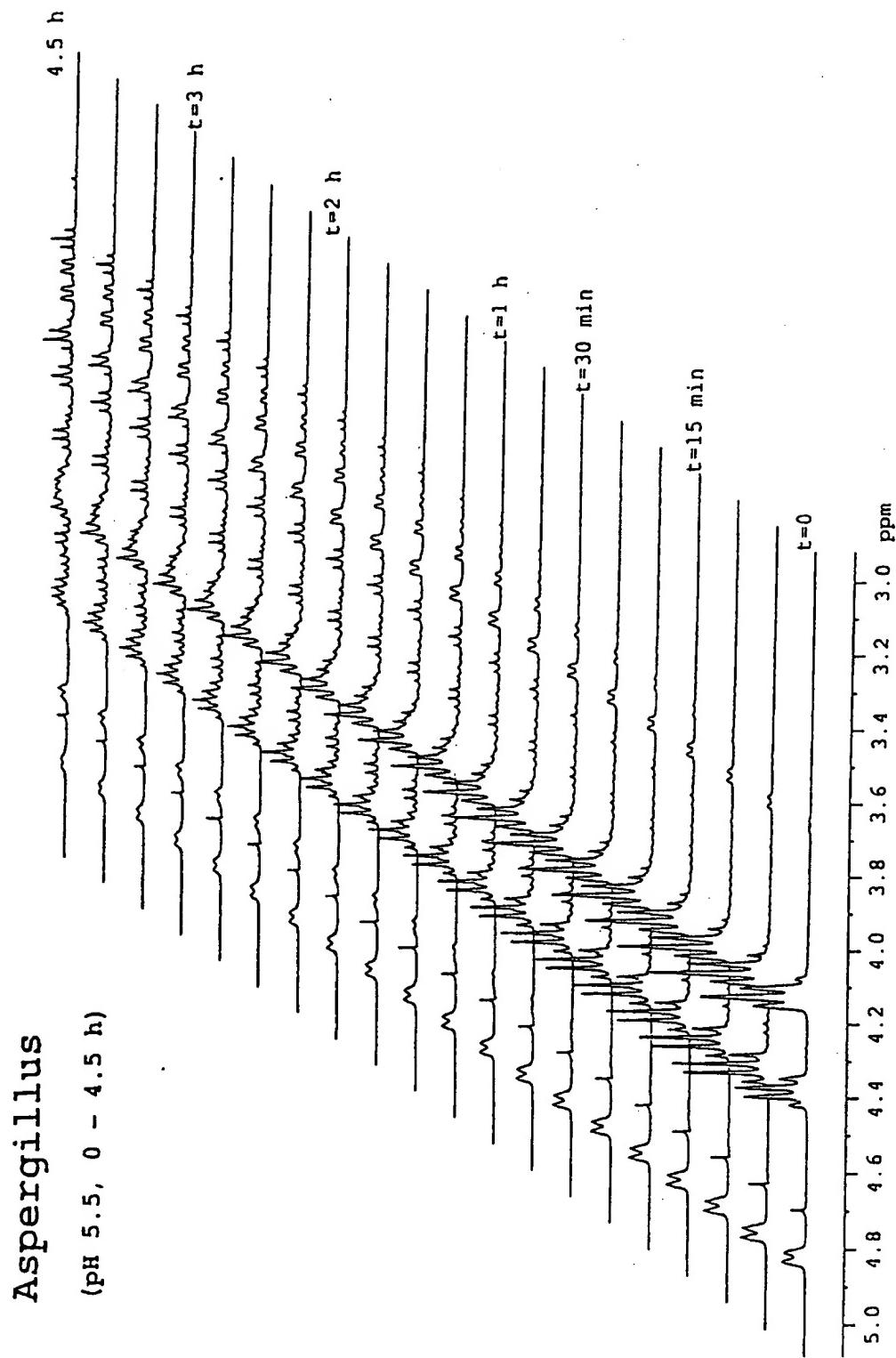
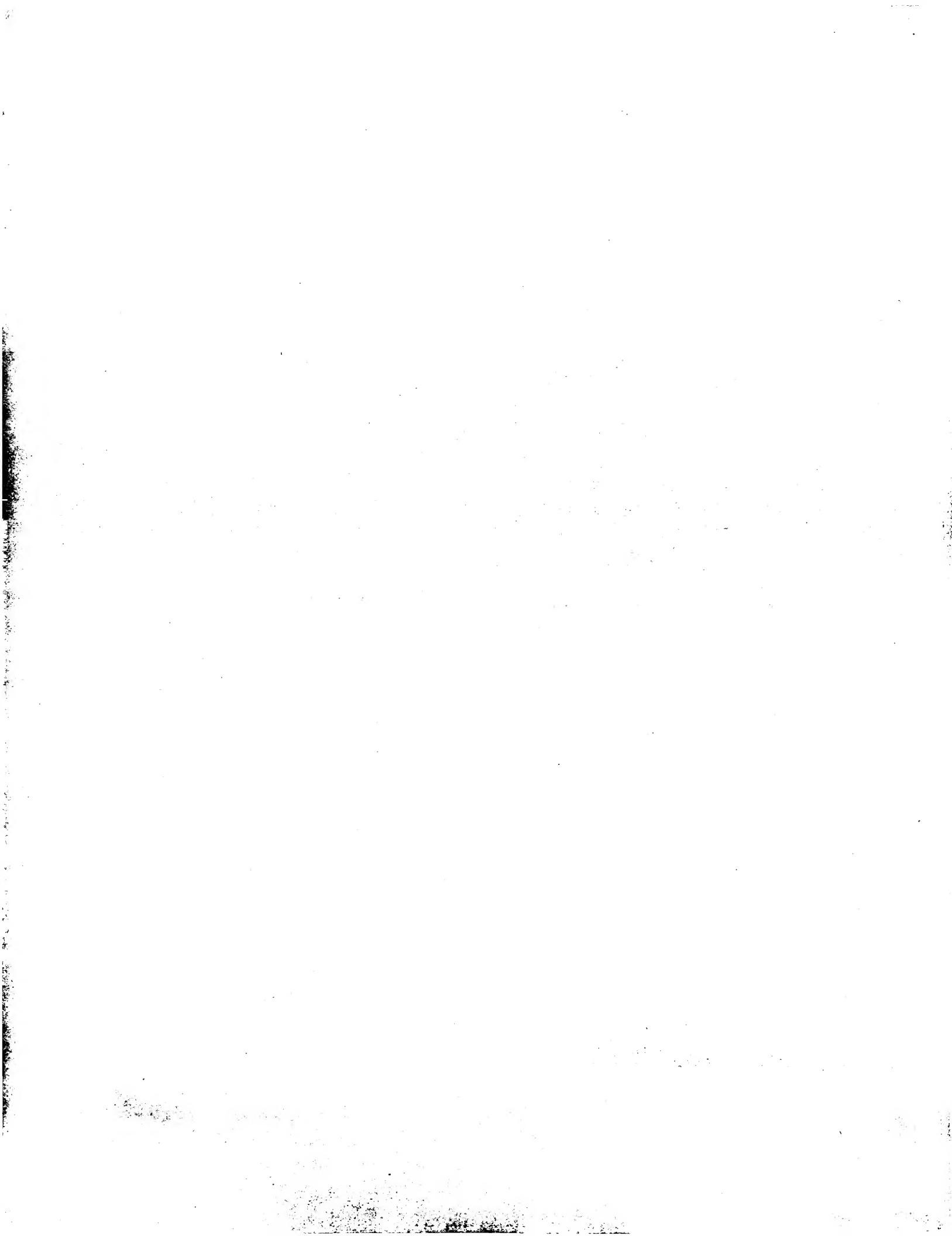


Fig. 15



16/38

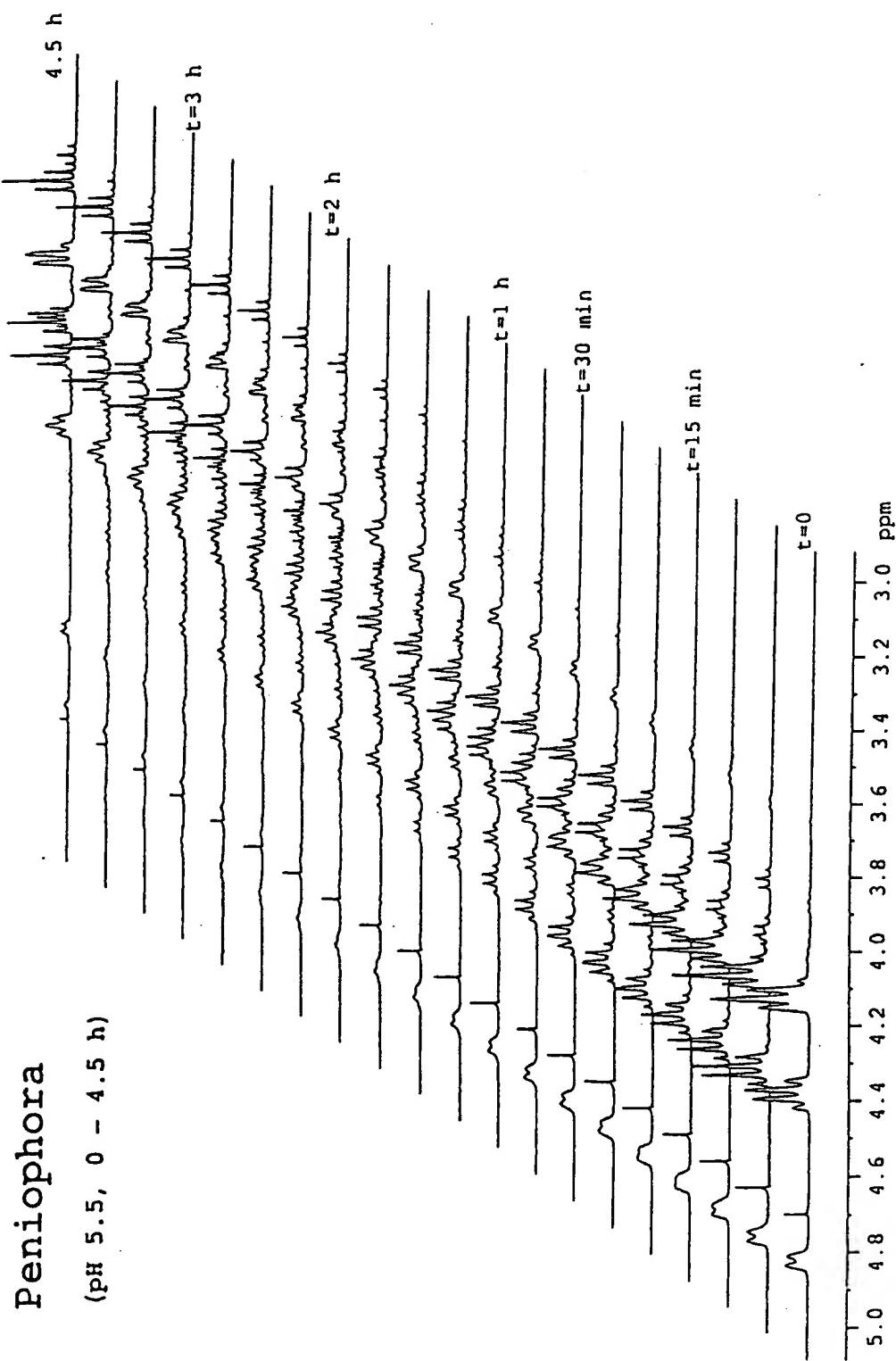
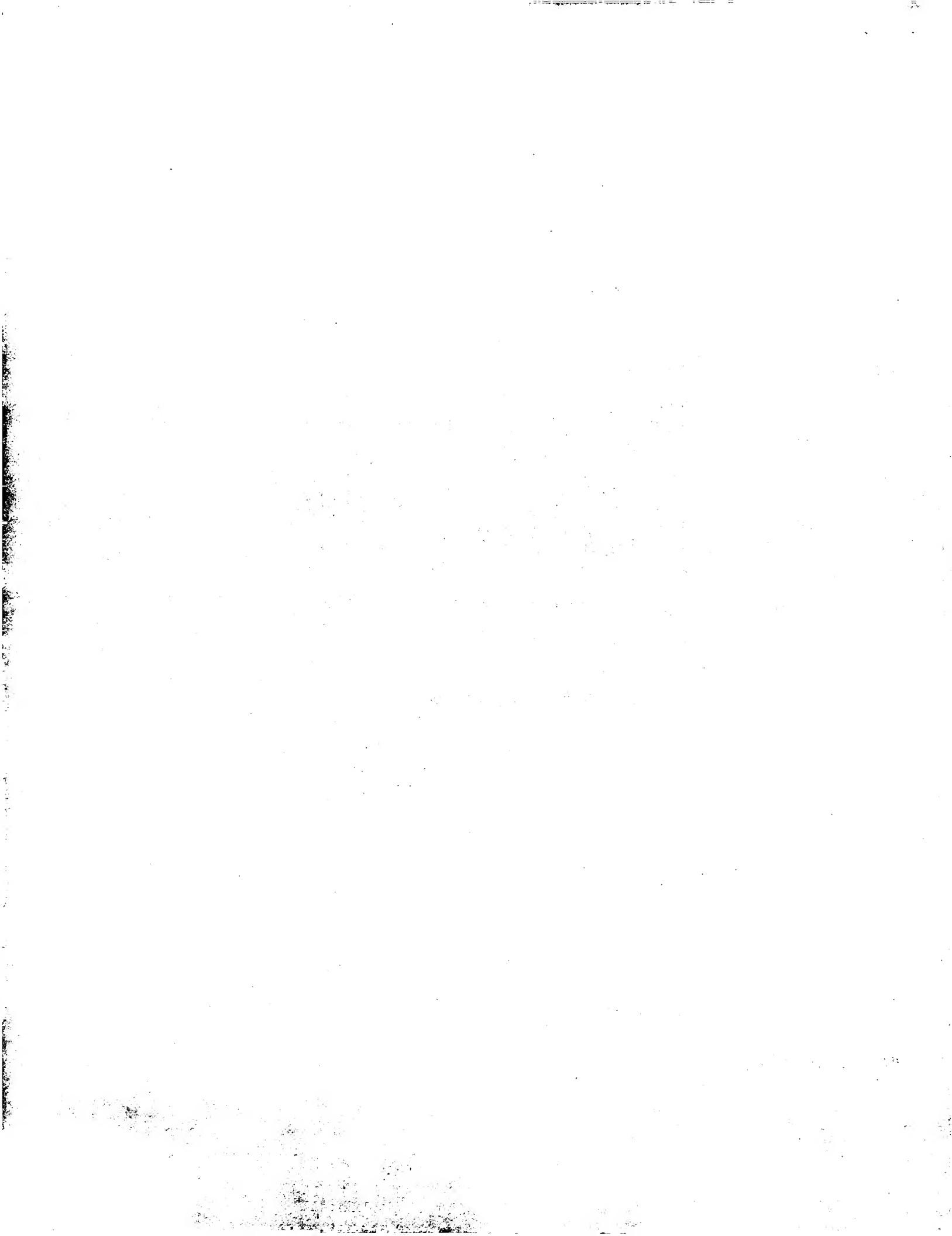


Fig. 16



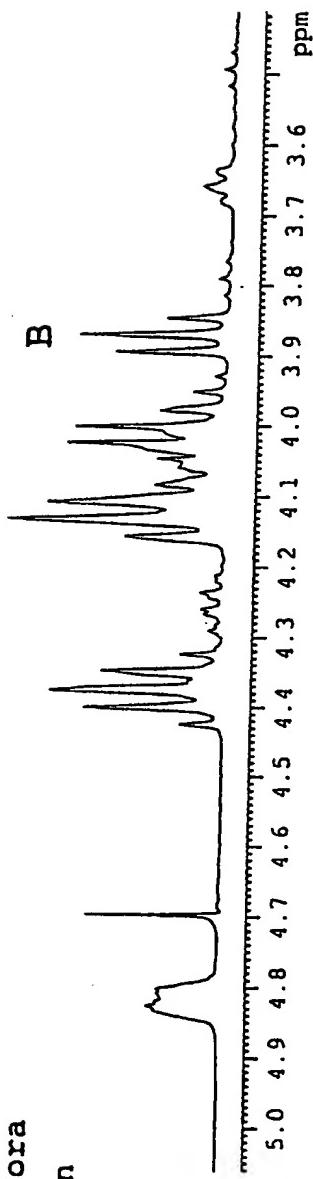
17/38

pH 5.5

Peniophora

20 min

- A Ins(1,2,4,5,6)P5 (H-3)
- B Ins(1,2,3,4,5)P5 (H-6)
- C Ins(1,2,5,6)P4 (H-3)



Aspergillus  
20 min

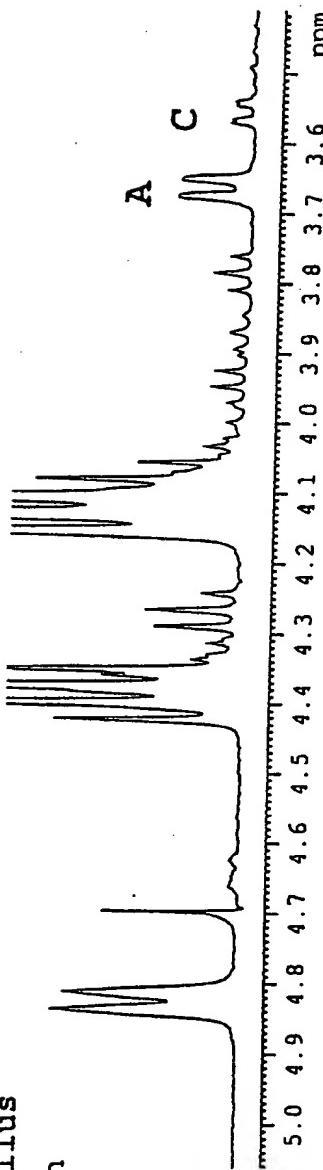
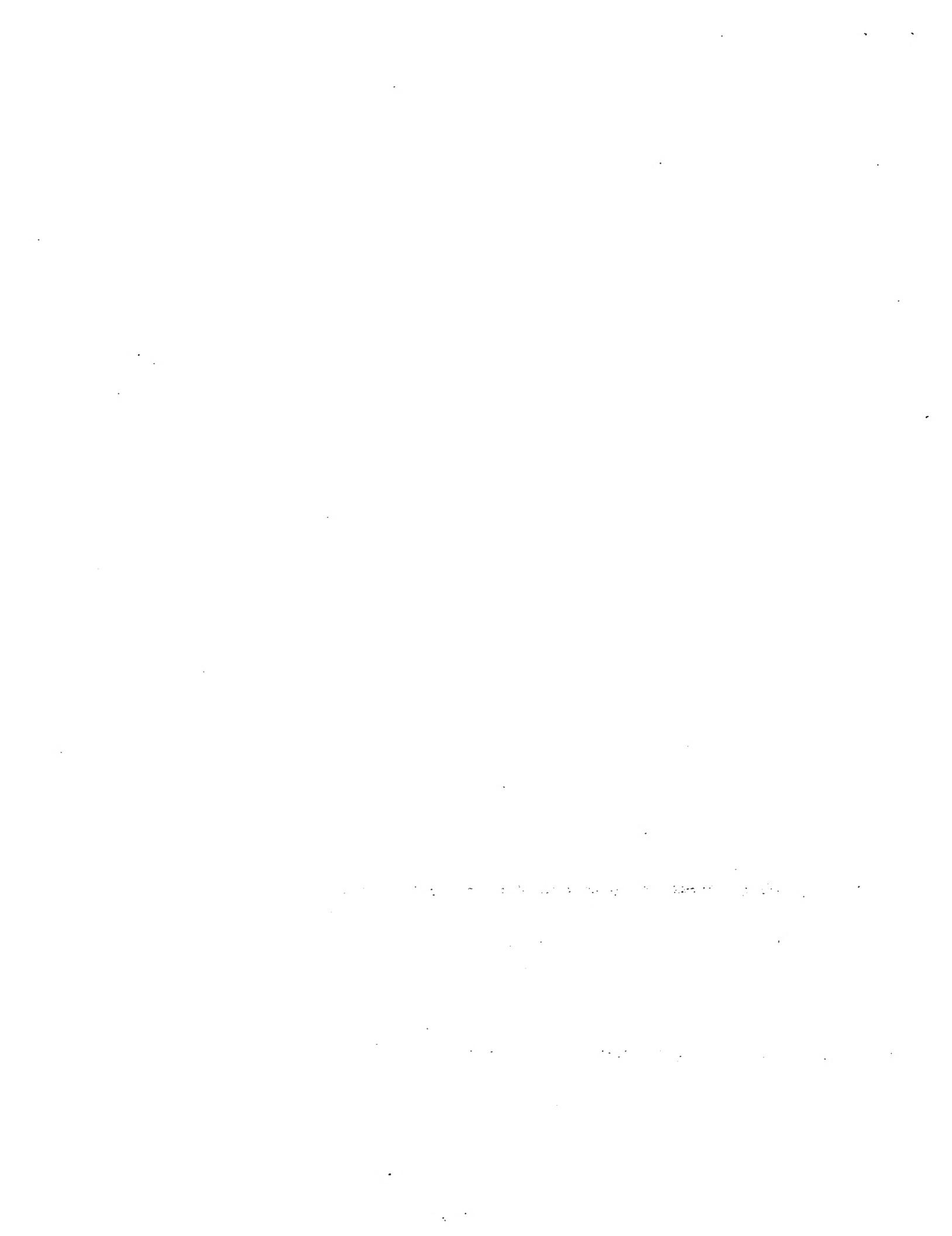


Fig. 17



18/38

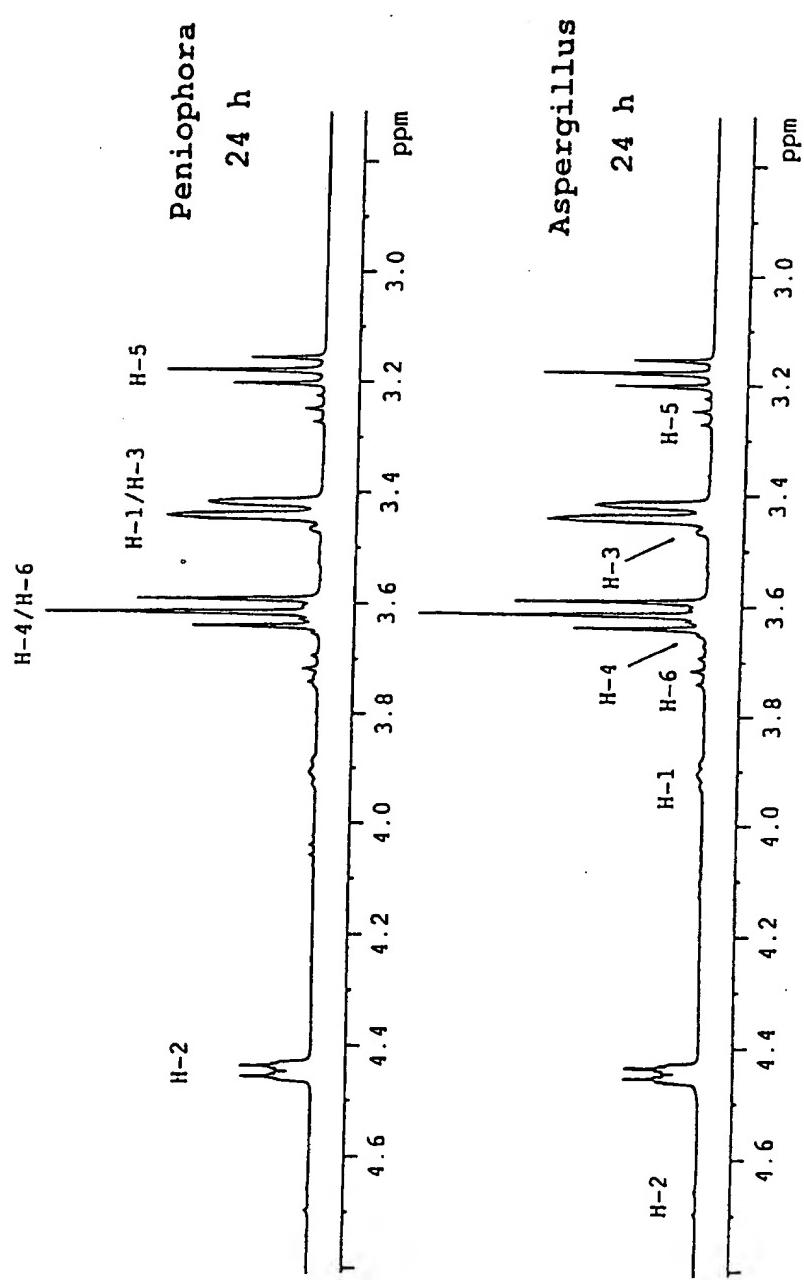
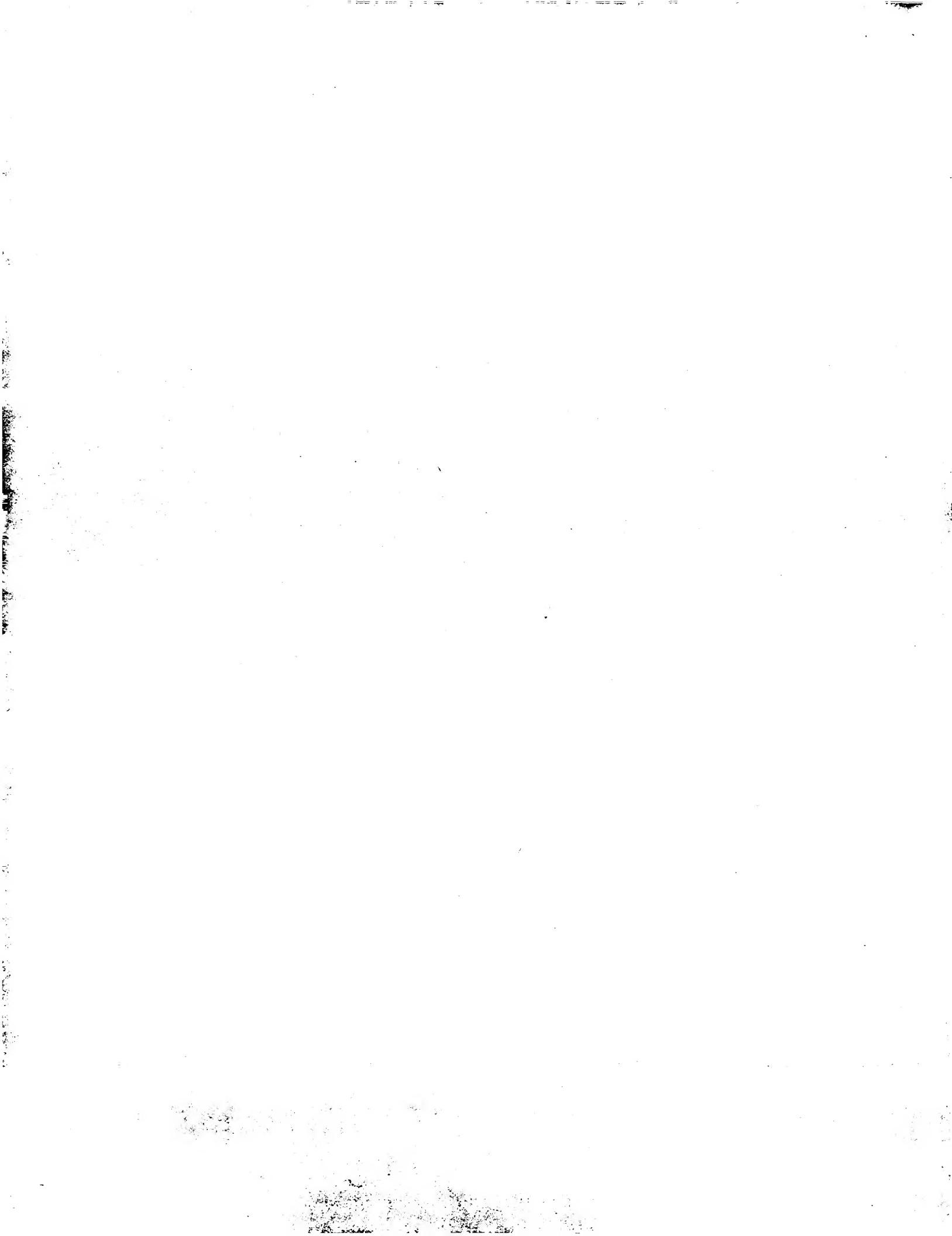


Fig. 18



19/38

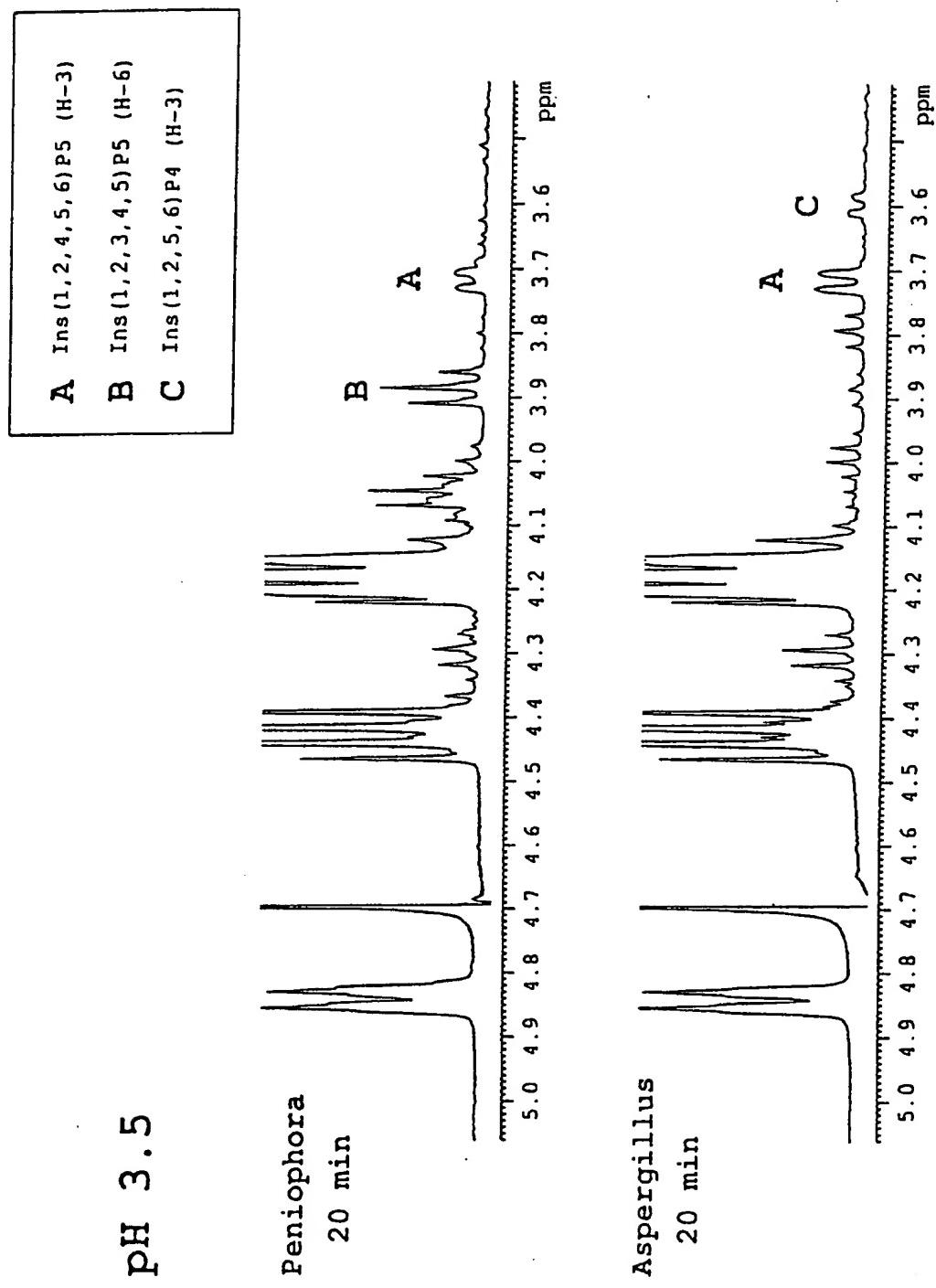
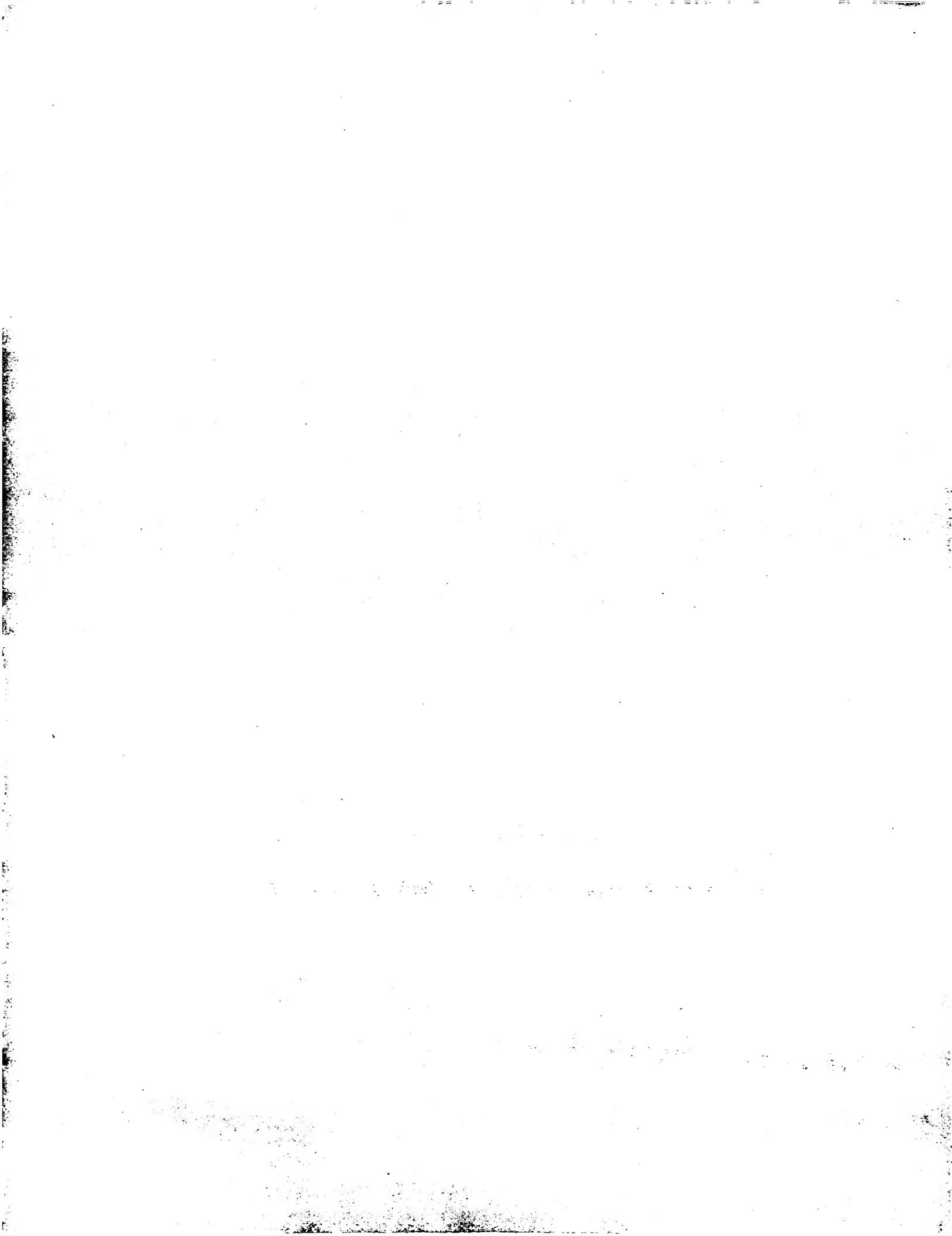


Fig. 19



20/38

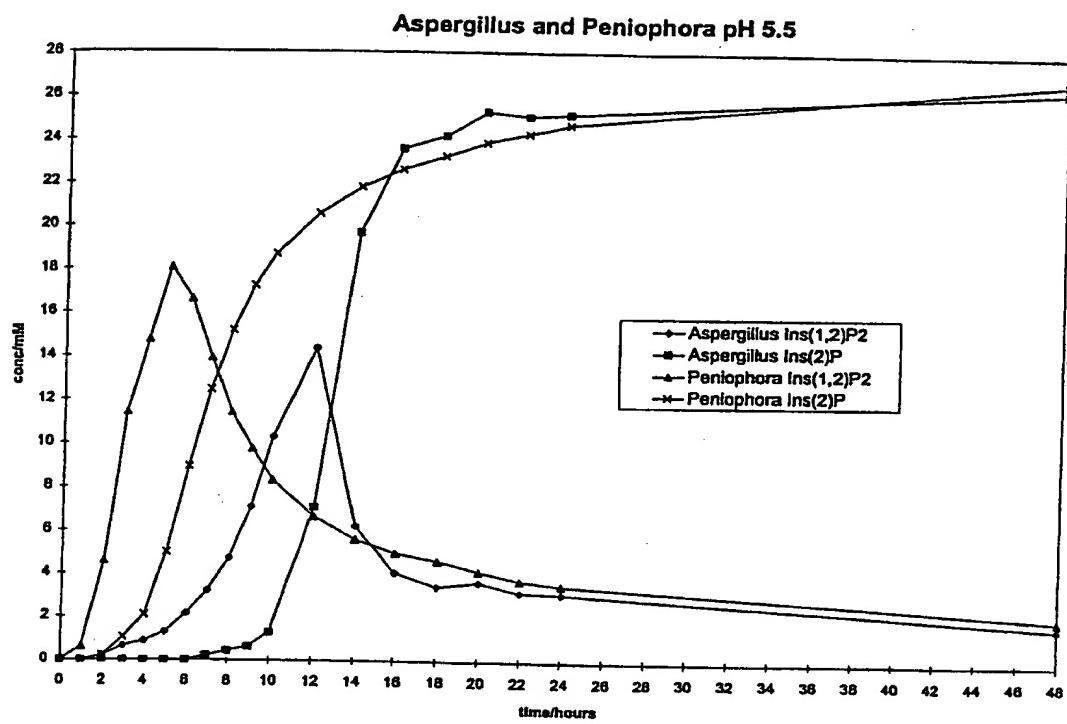
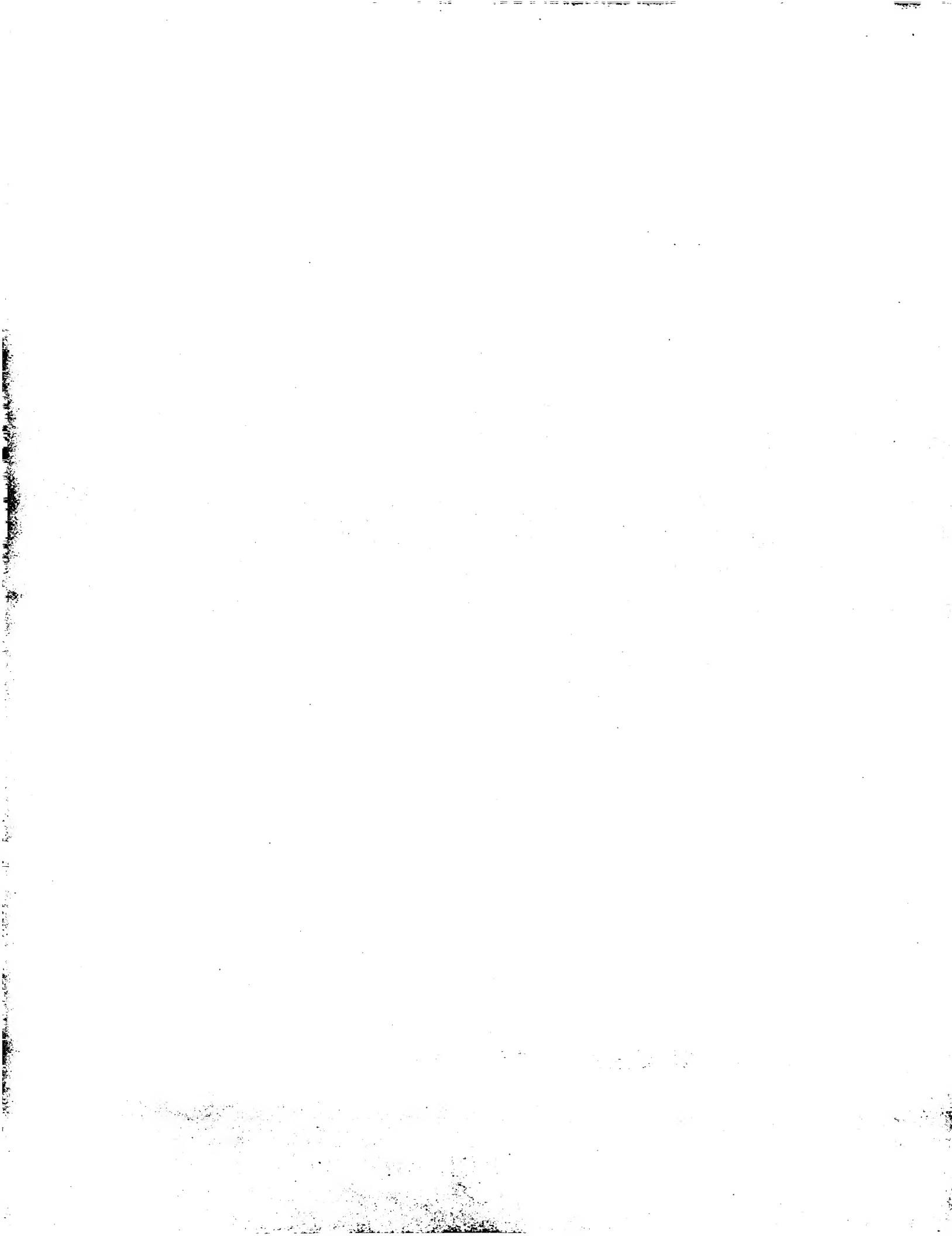


Fig. 20



21/38

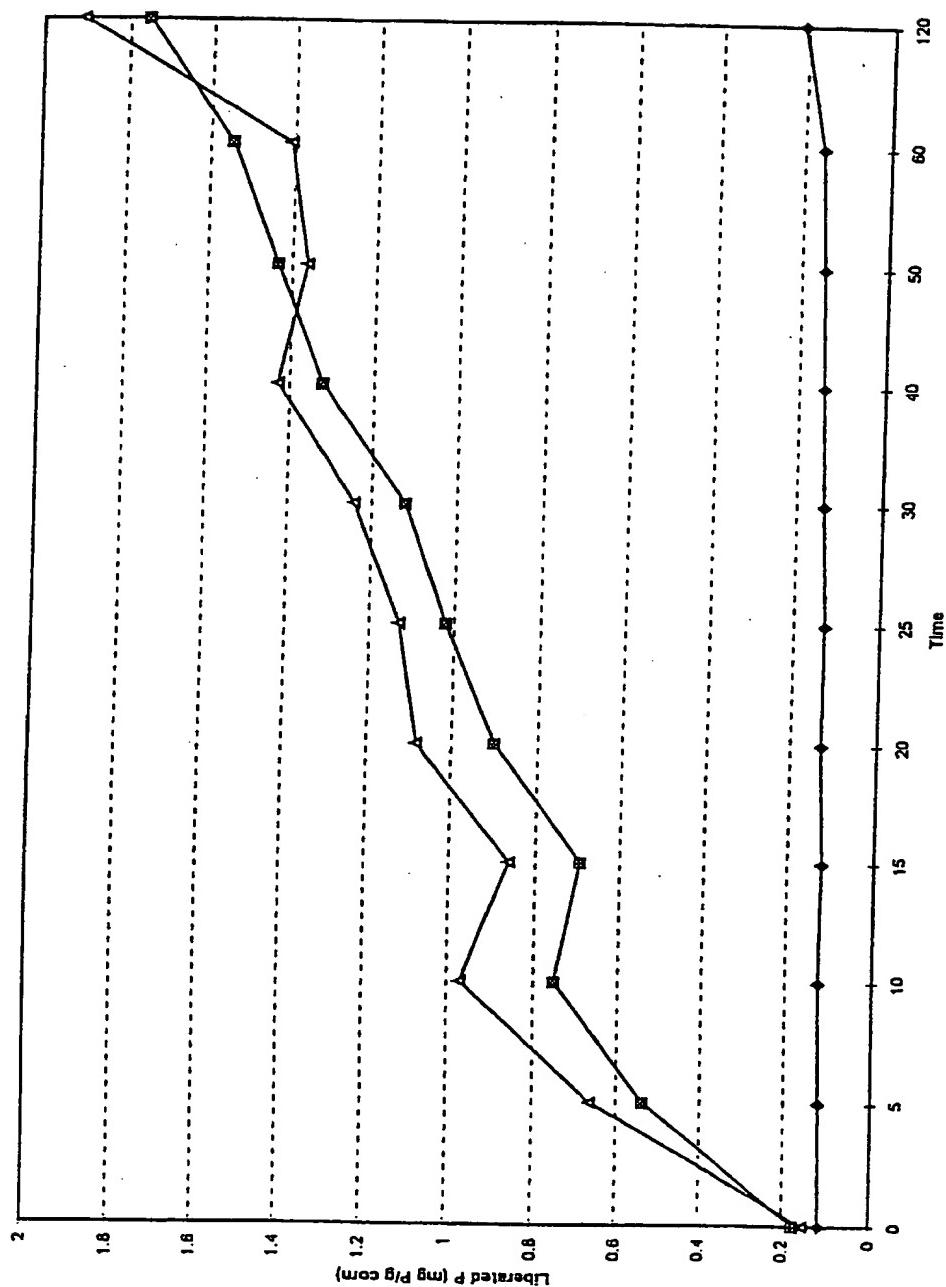
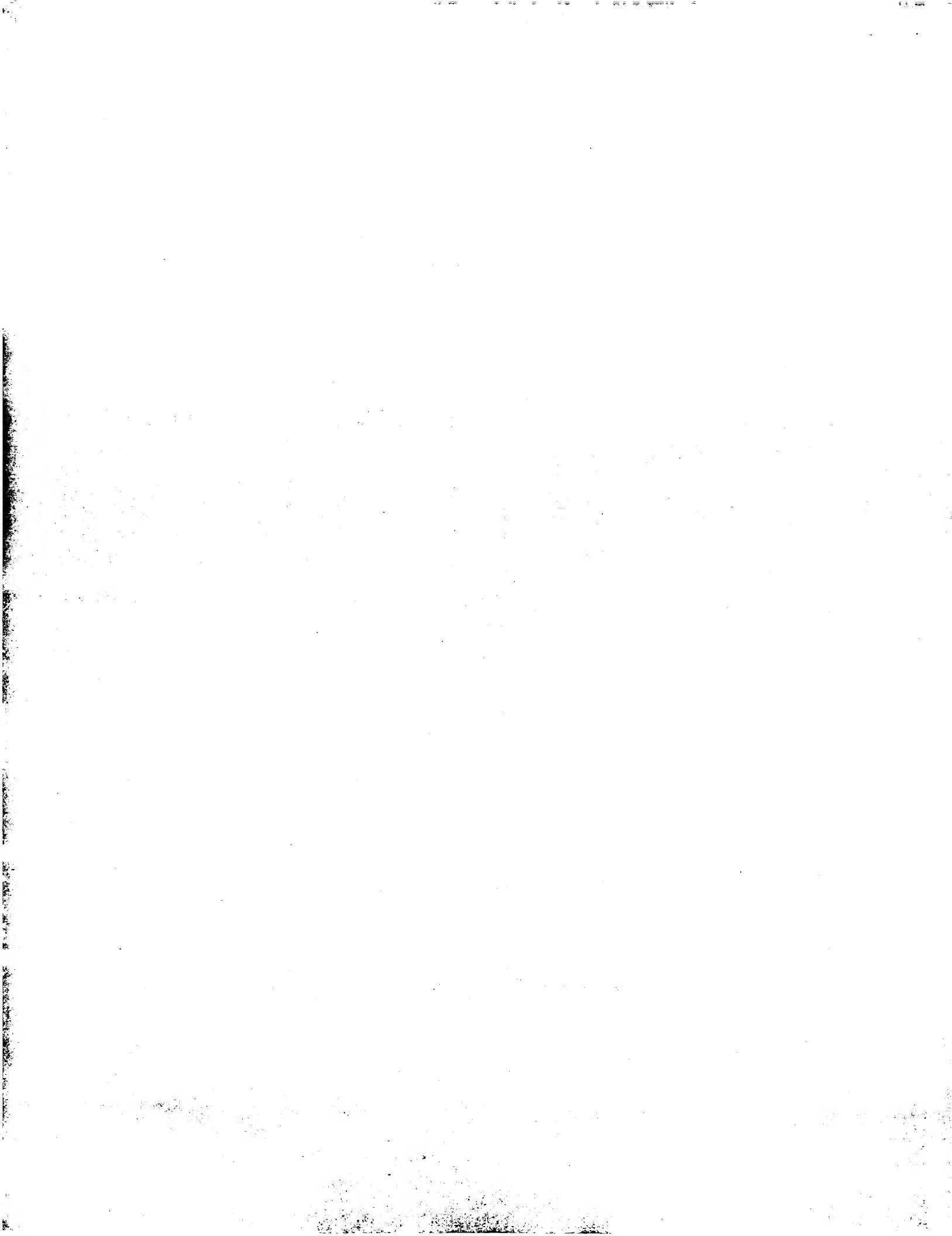


Fig. 21



22/38

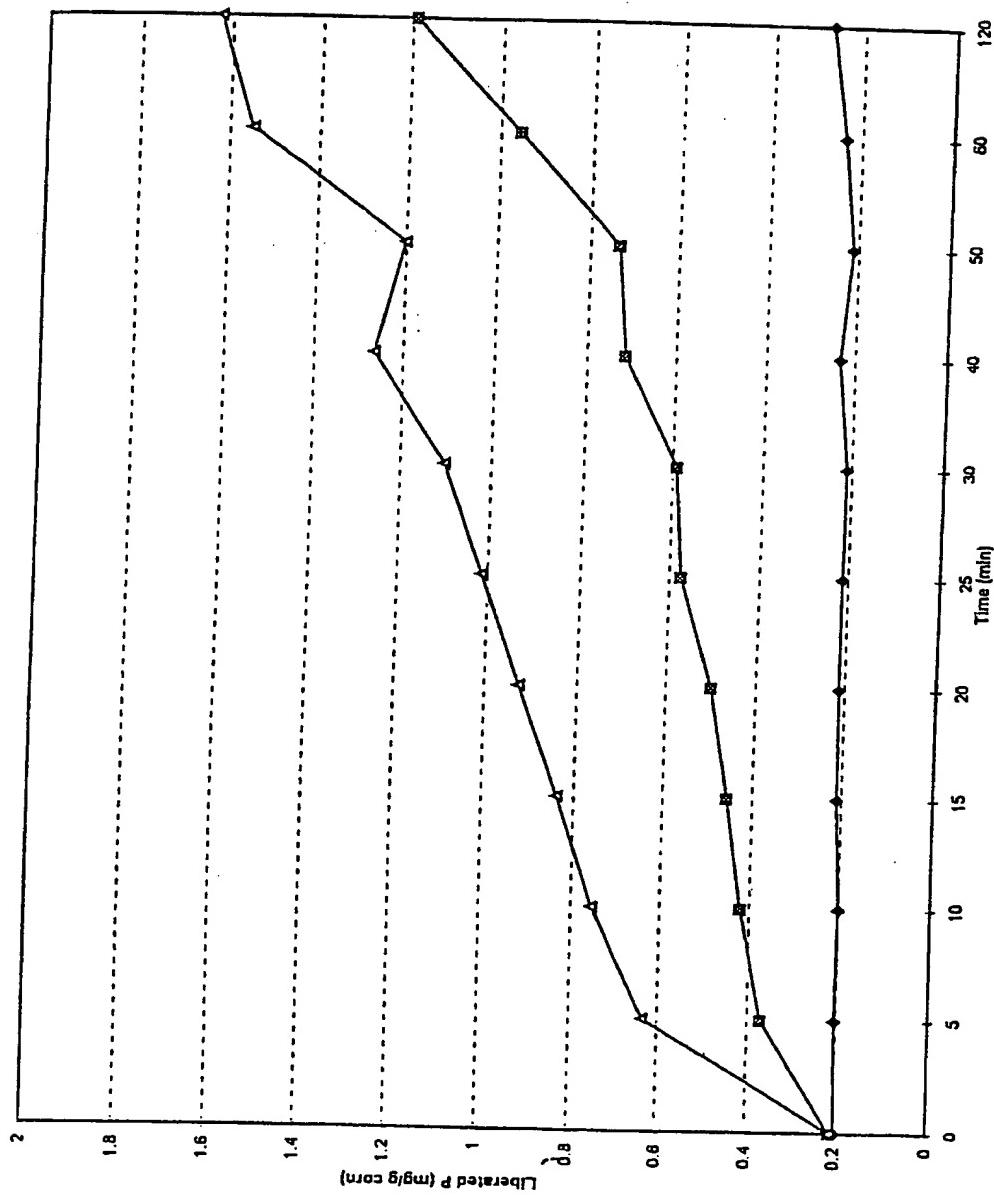
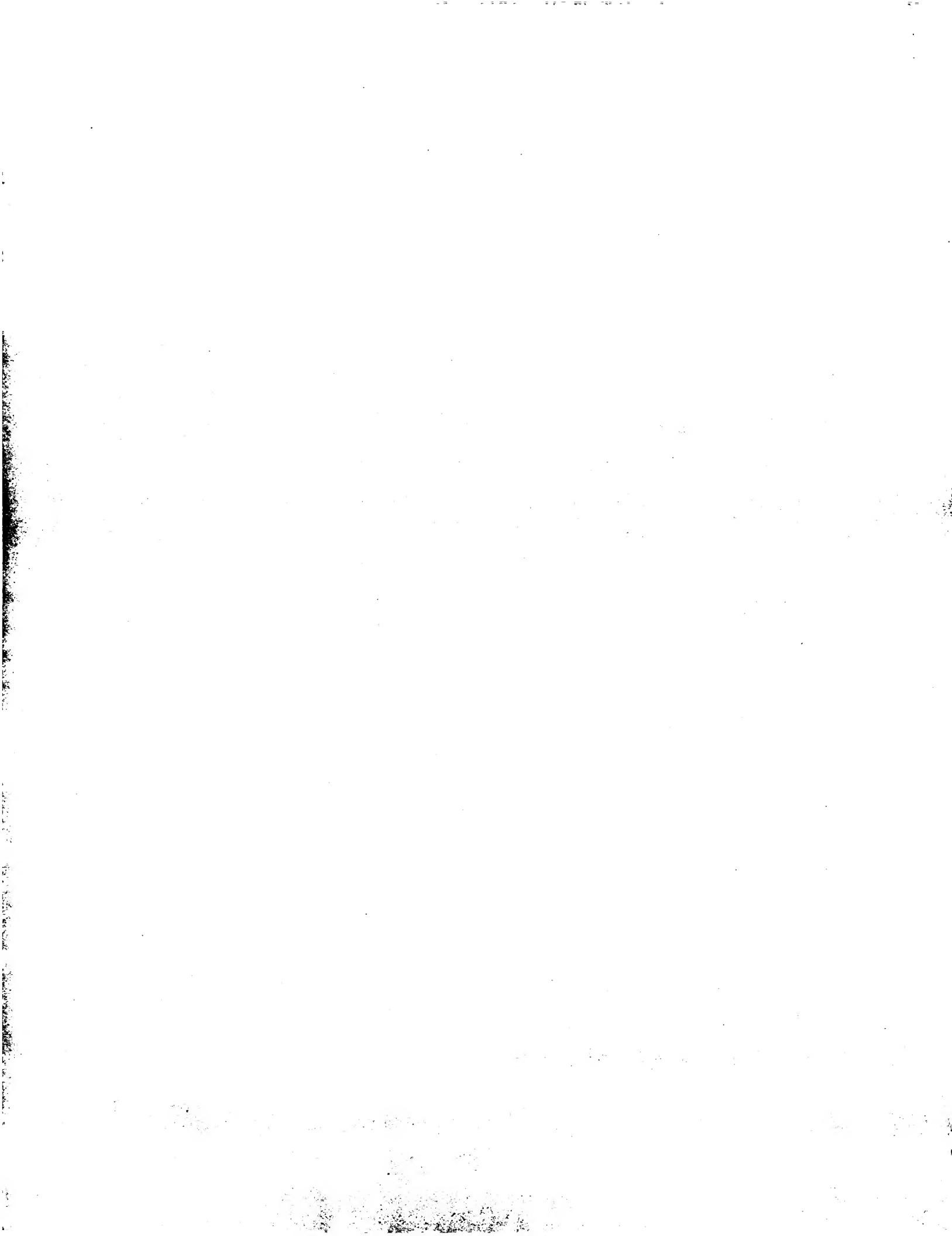


Fig. 22



23/38

**Agrocybe phytase pH-profile, 5 mM phytate, 30 min at 37 °C**

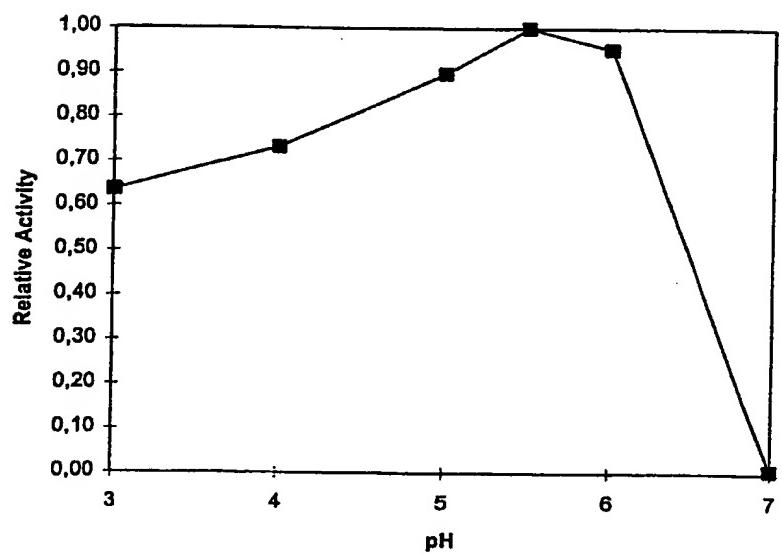
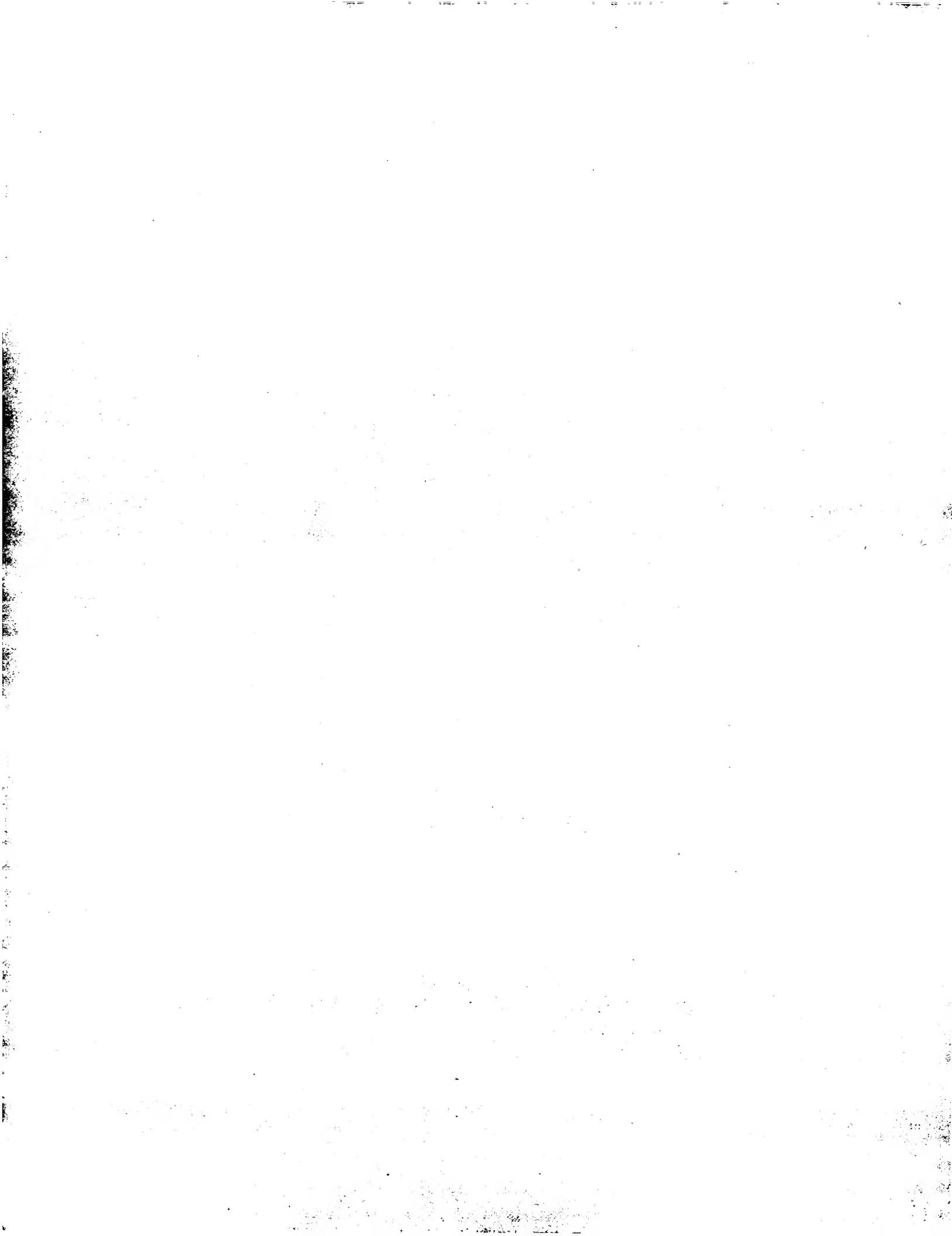


Fig. 23



24/38

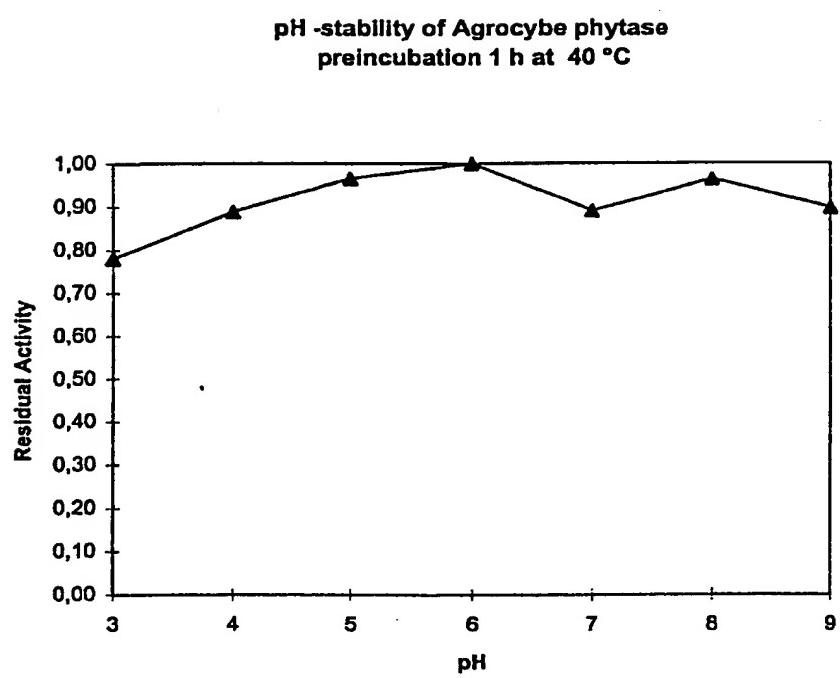


Fig. 24



25/38

Temperature profile of Agrocybe phytase in 0.1 M  
sodium acetate, 5mM phytate, pH 5.5

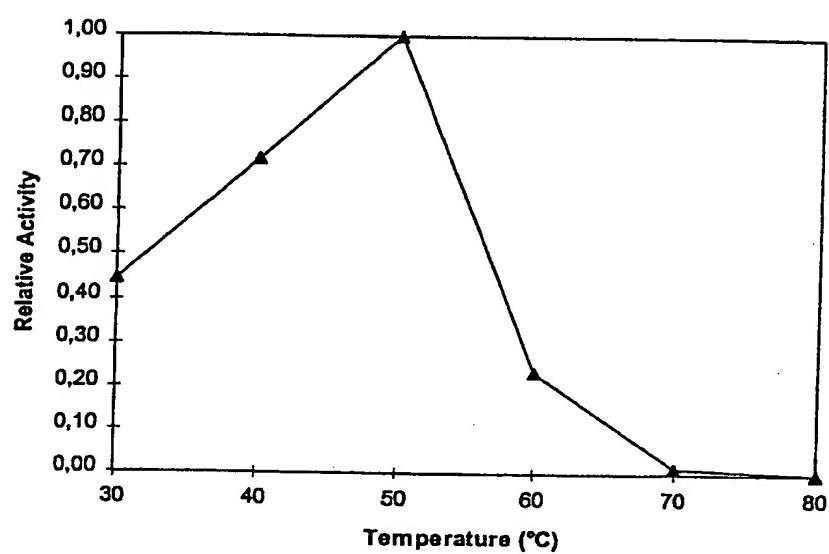
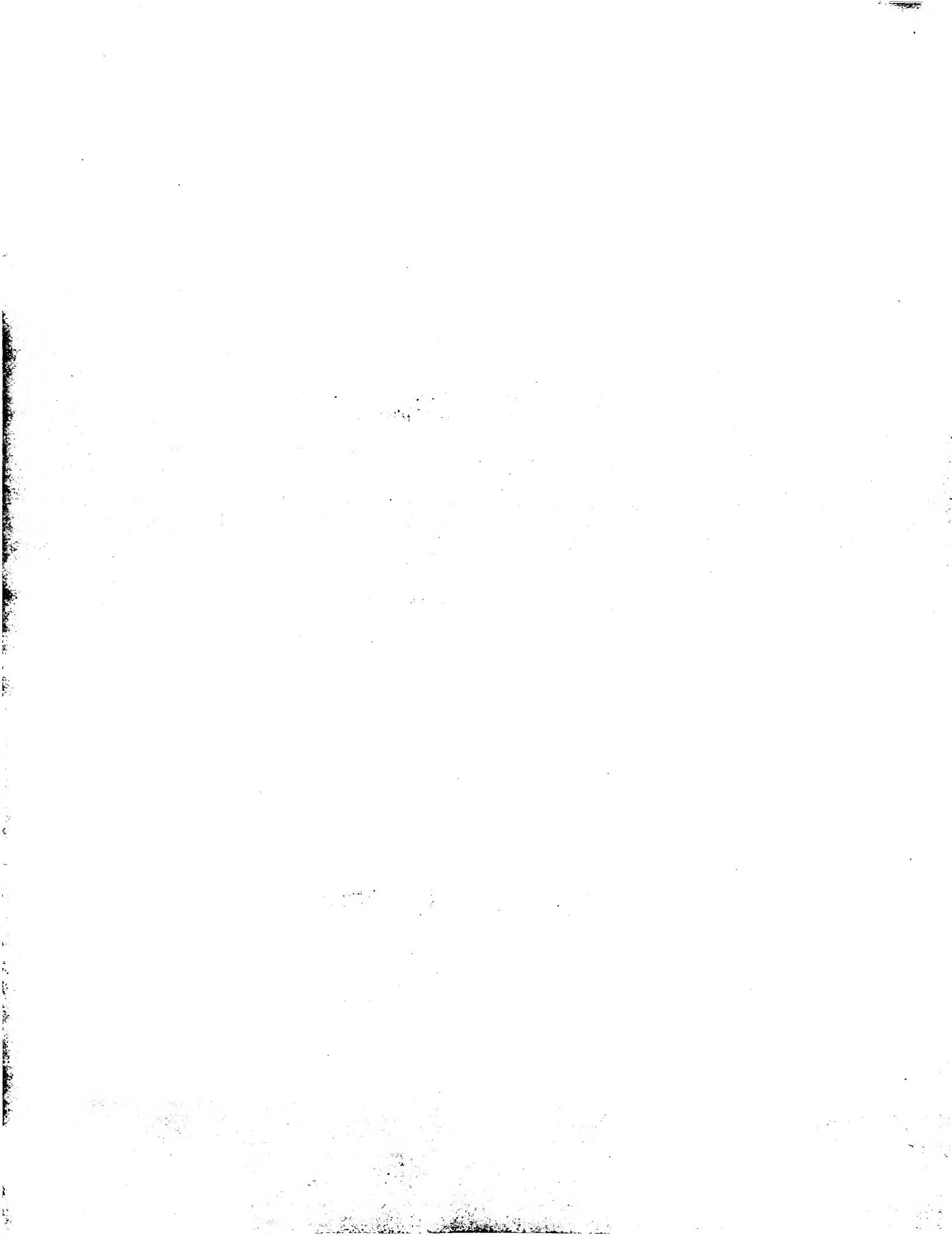


Fig. 25



26/38

**Temperature stability of Agrocybe phytase -  
preincubation 60 min in 0.1 M sodium acetate pH 5.5**

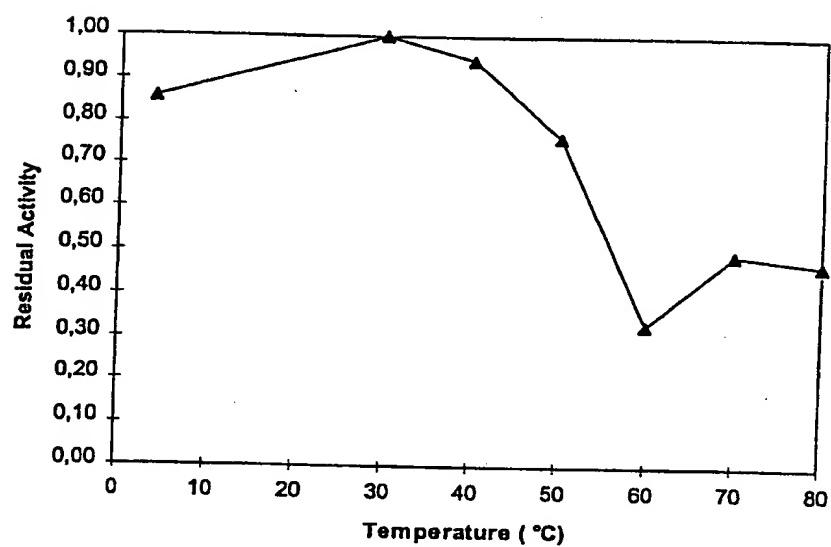
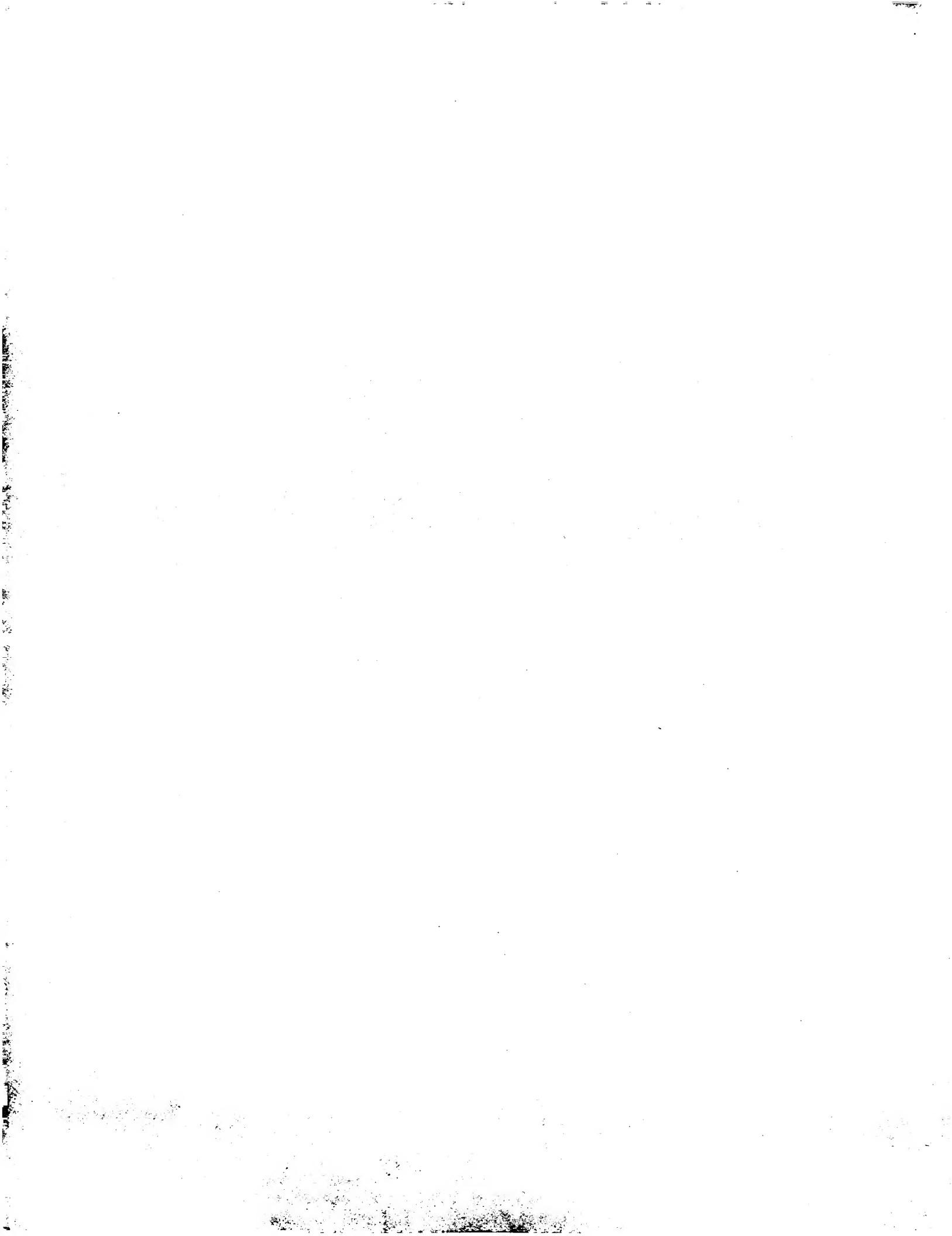


Fig. 26



27/38

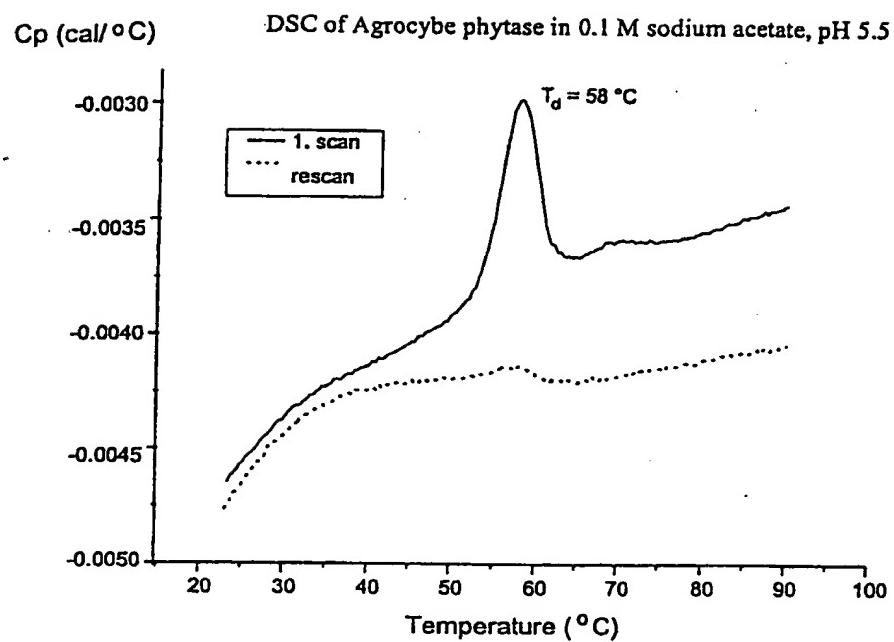
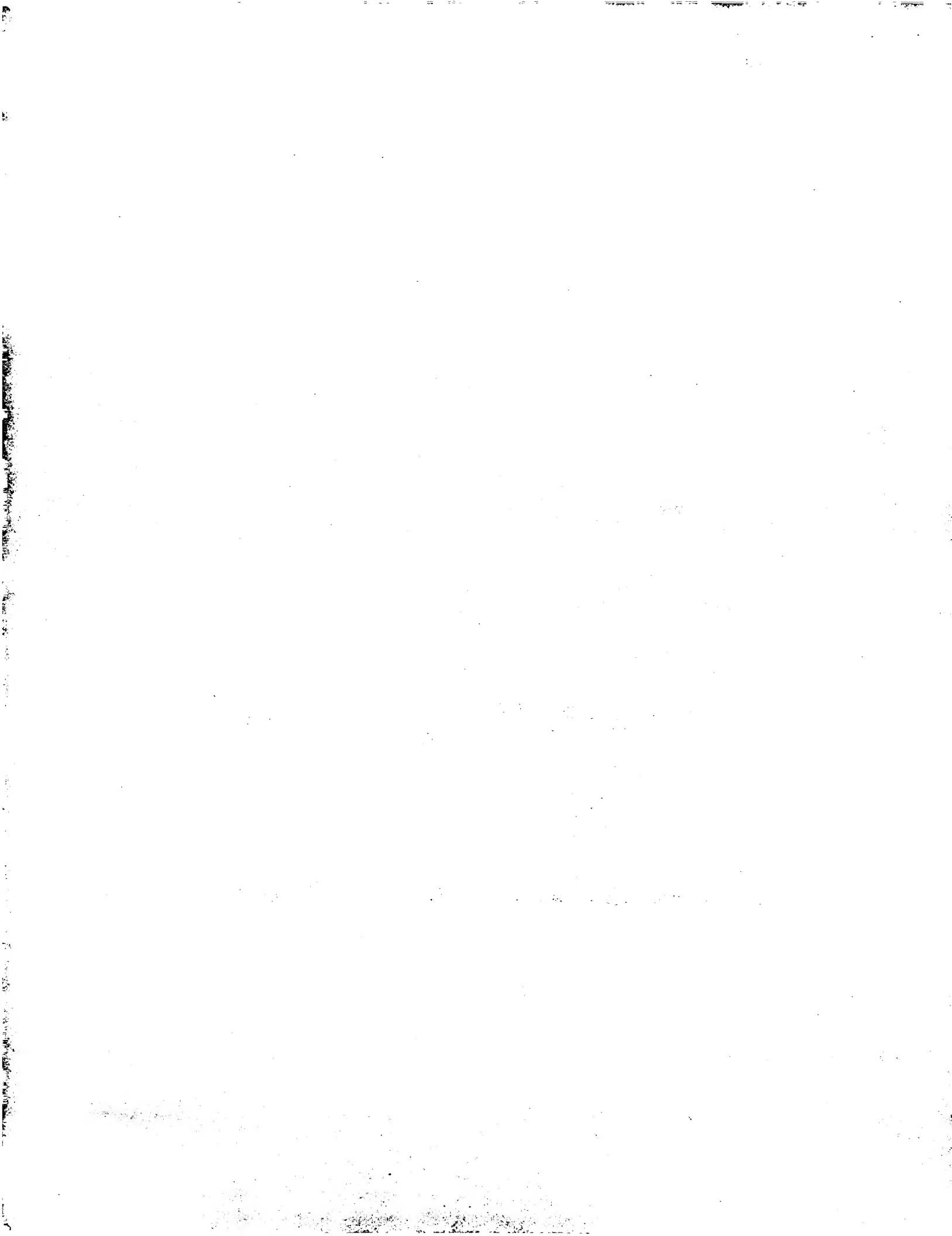


Fig. 27



28/38

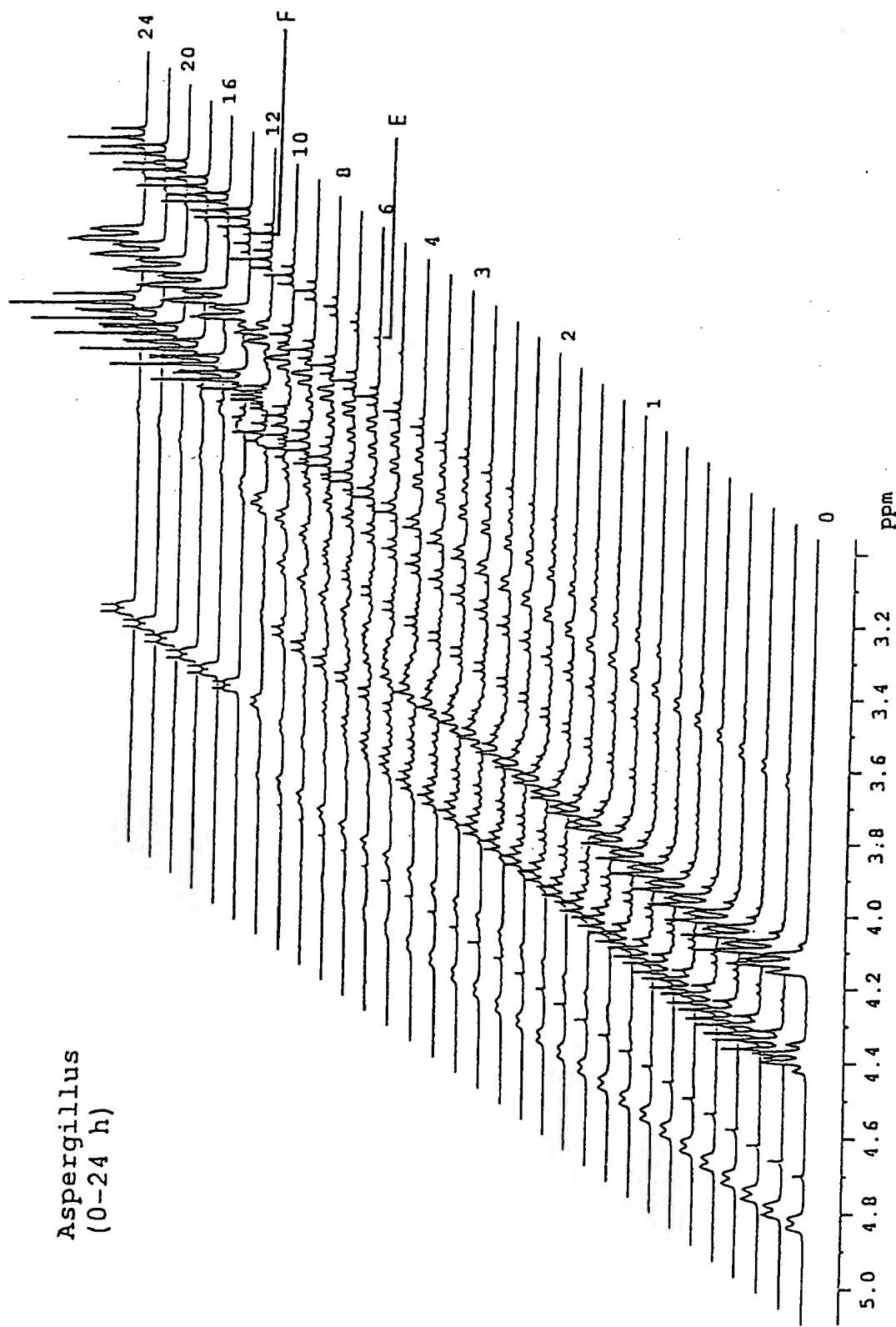
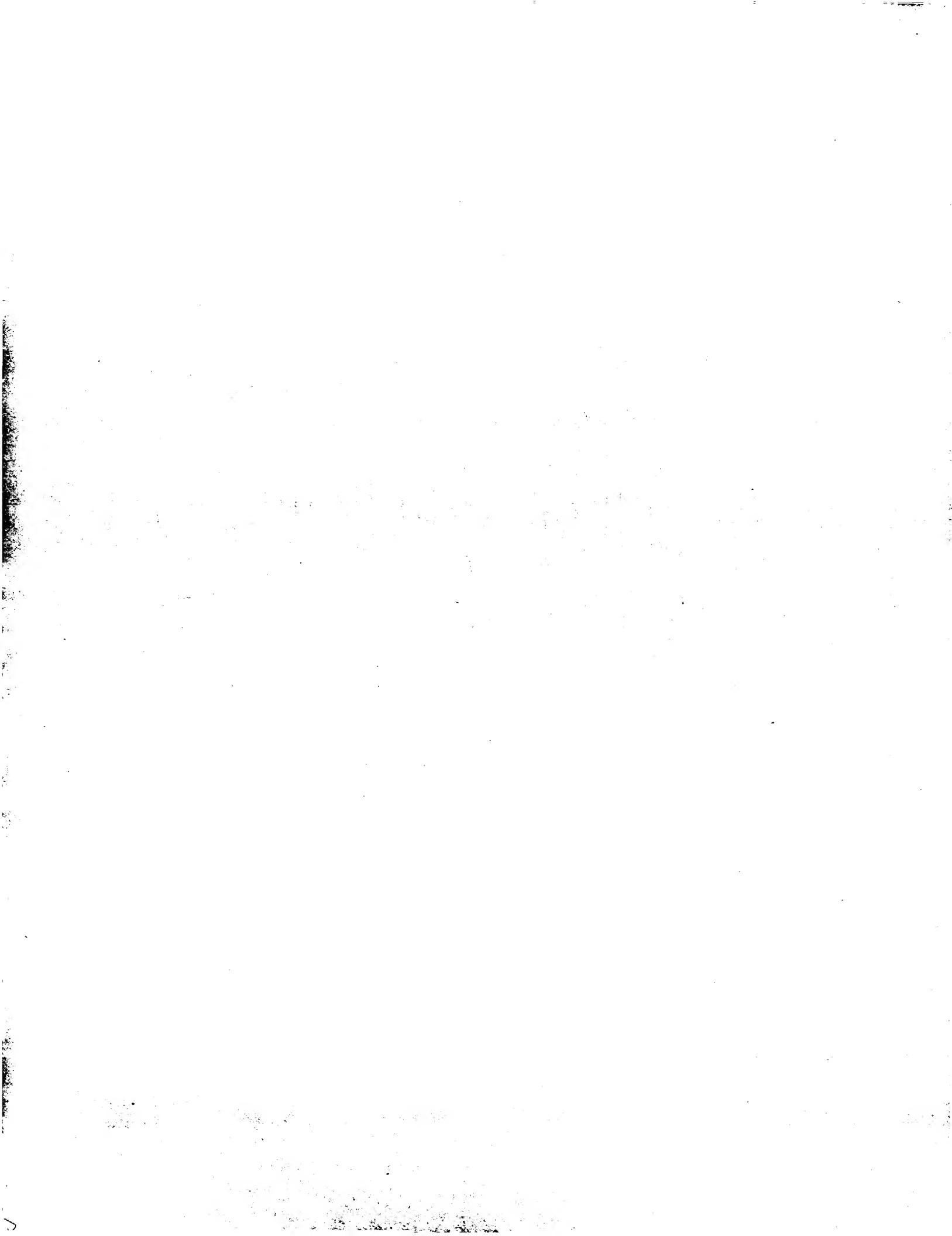


Fig. 28



29/38

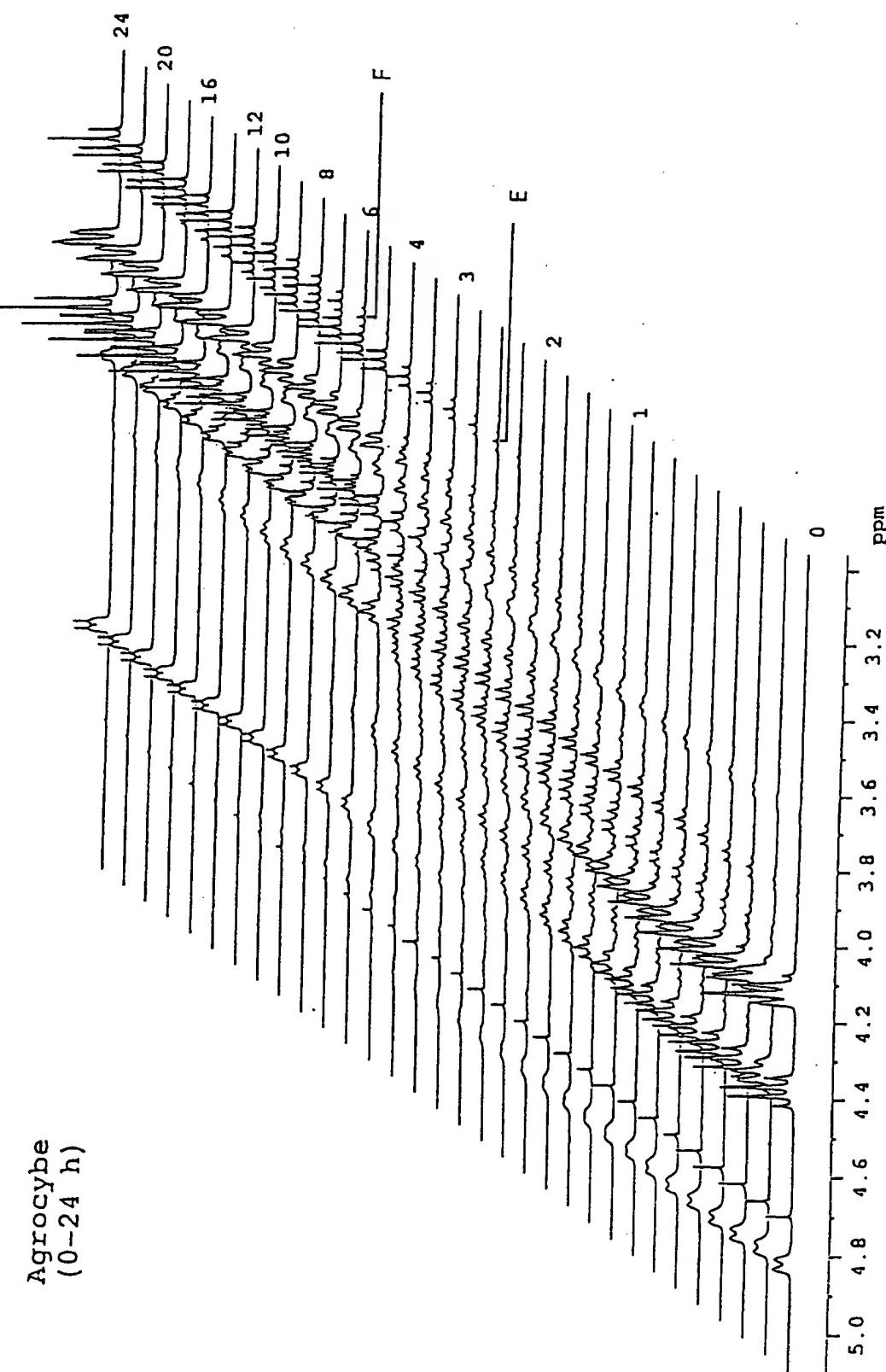


Fig. 29



30/38

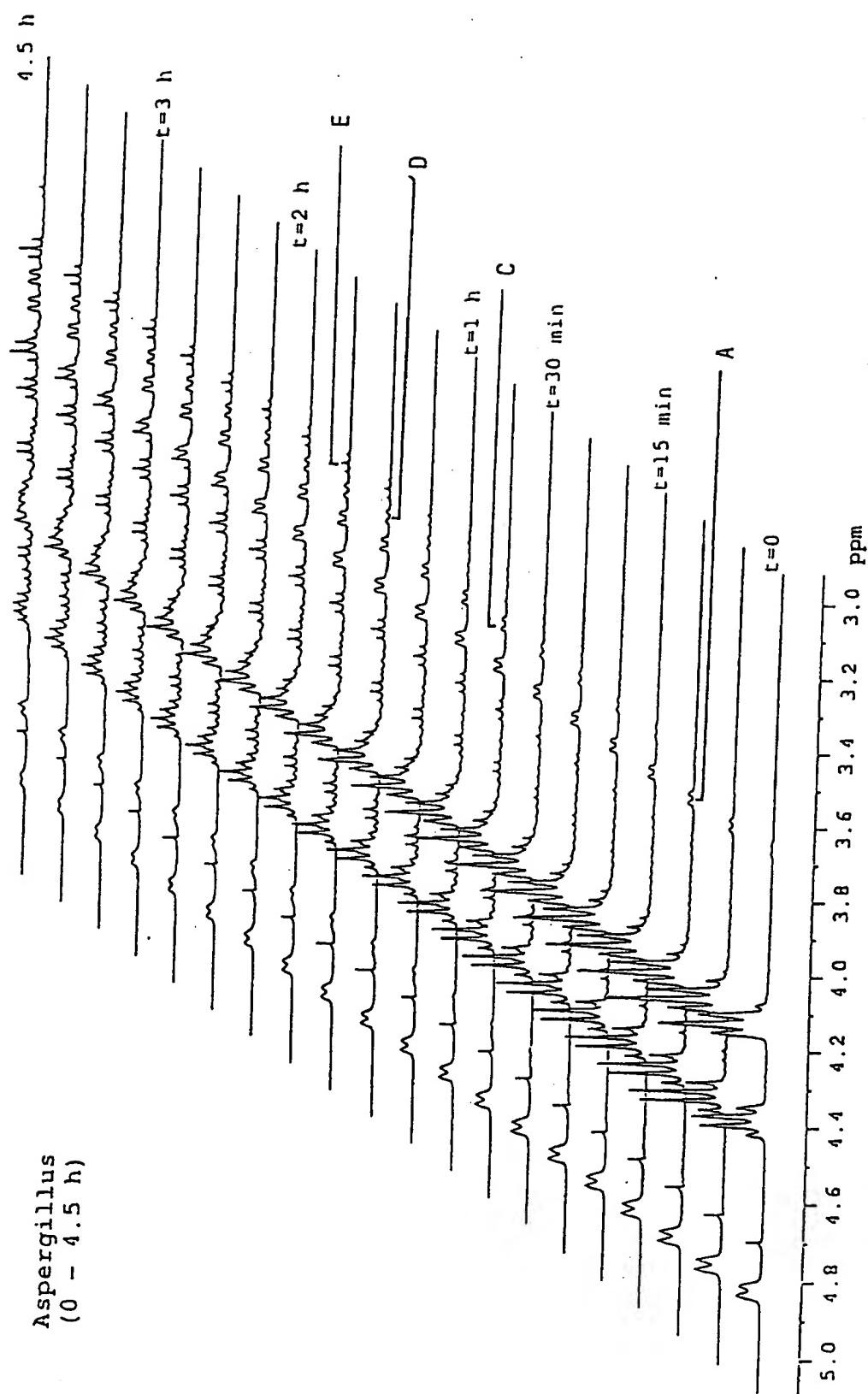


Fig. 30



31/38

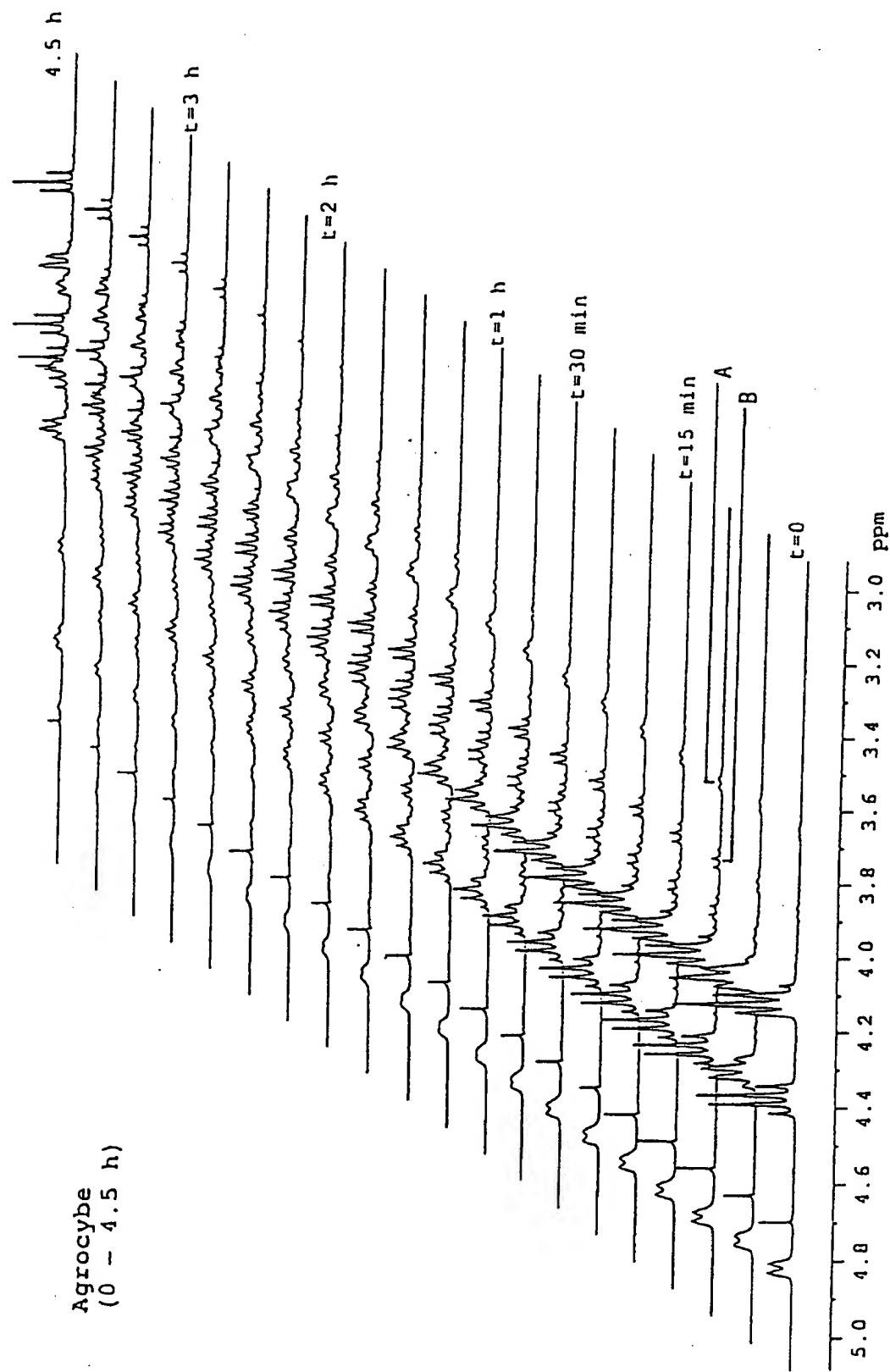
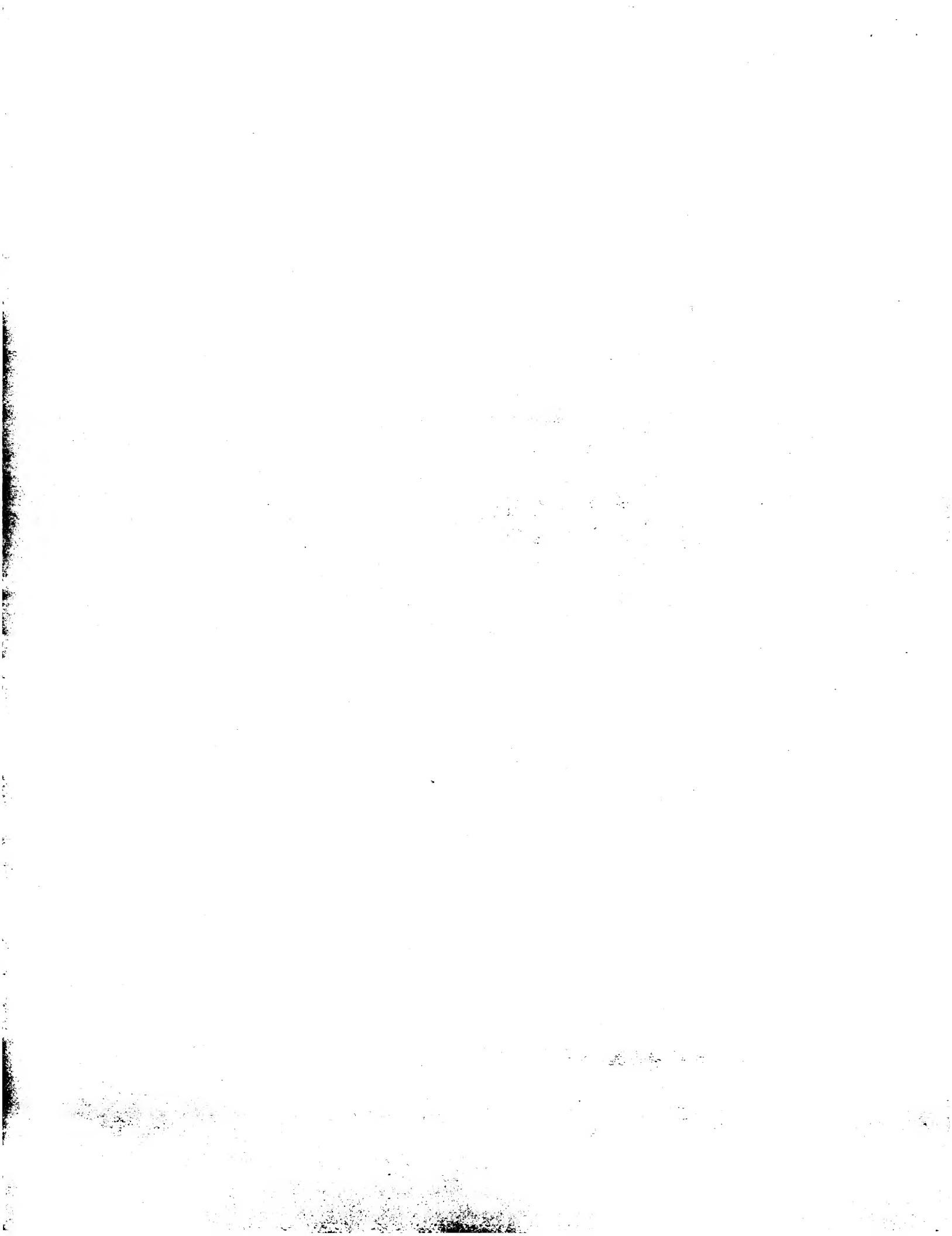


Fig. 31



32/38

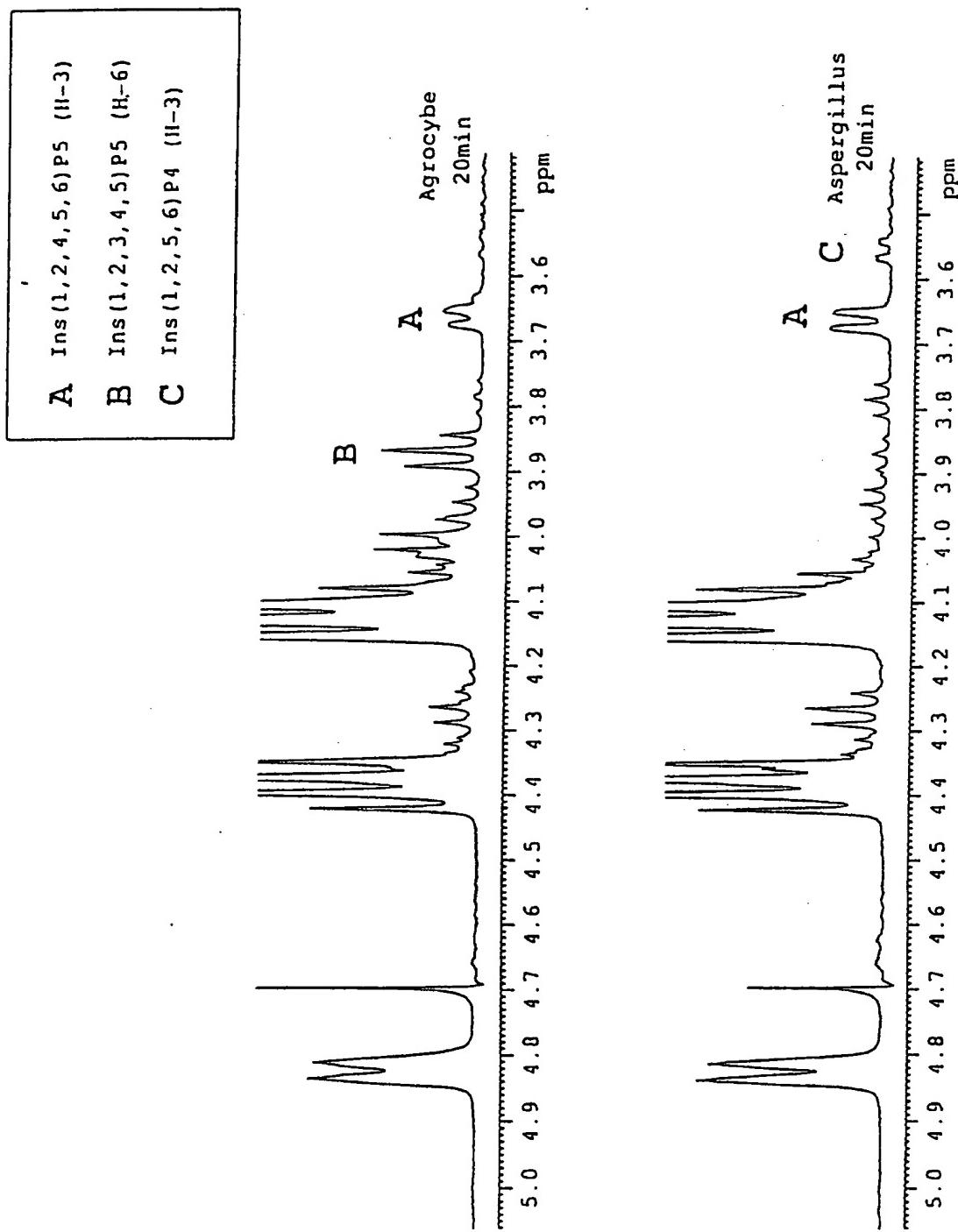


Fig. 32



33/38

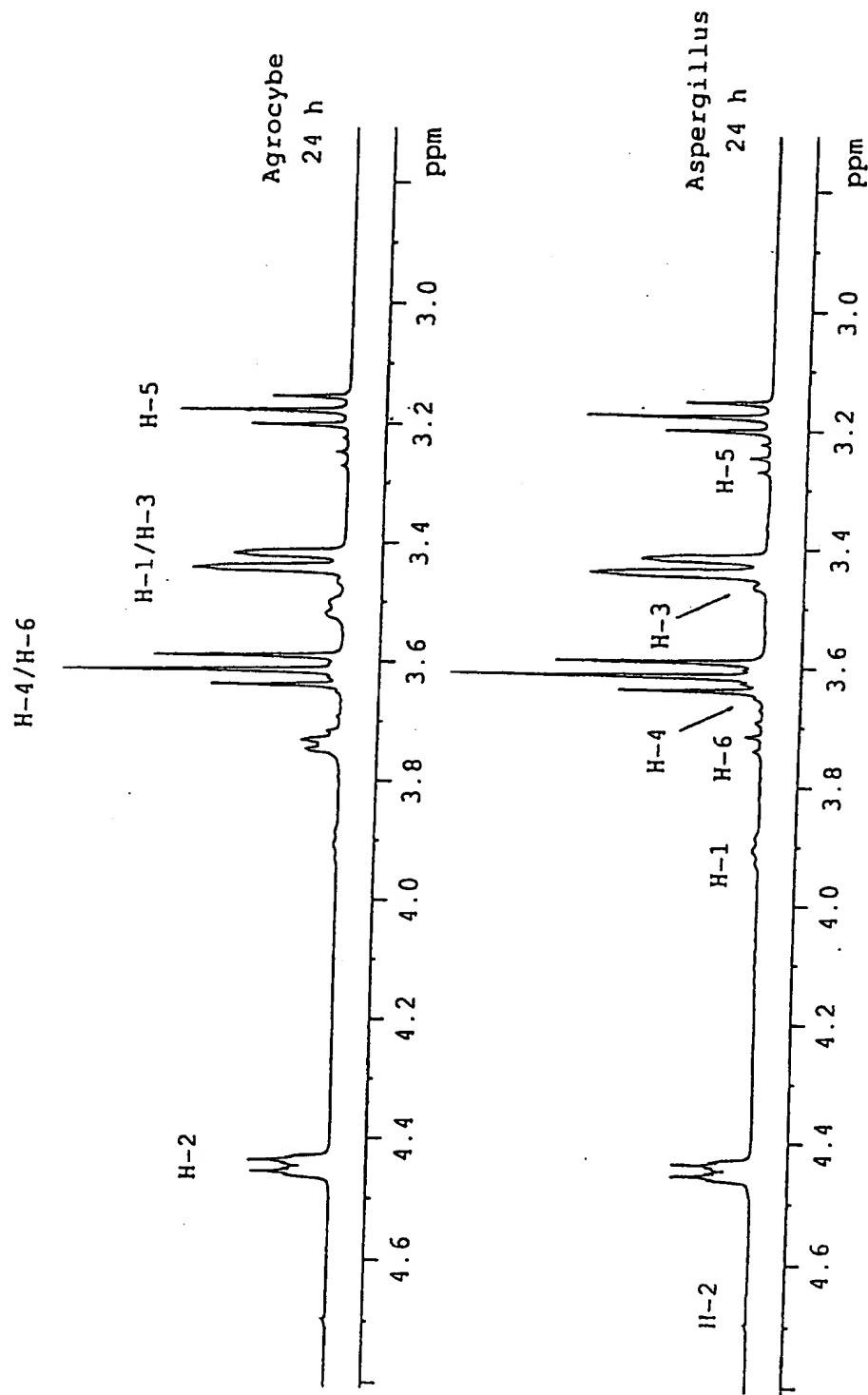
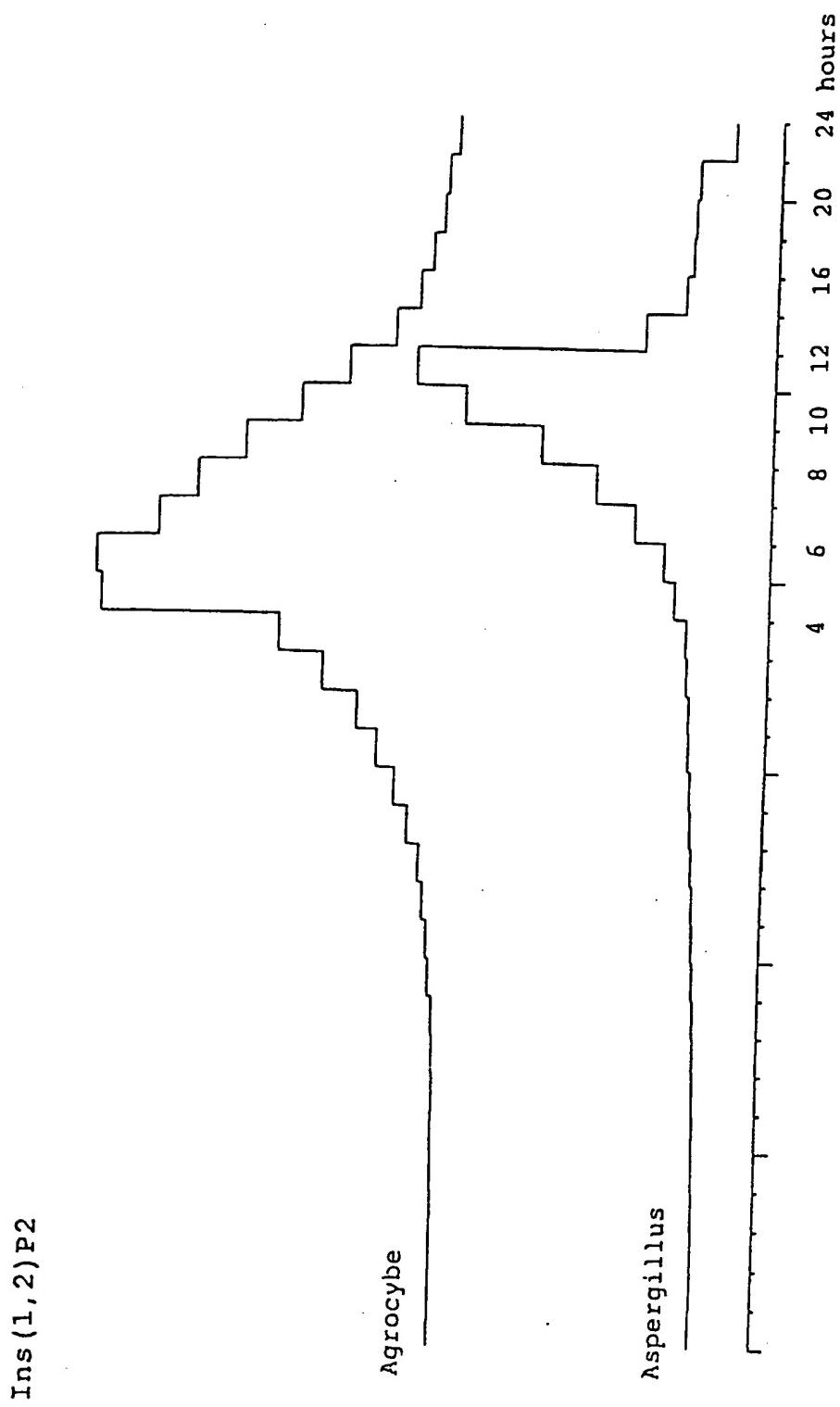


Fig. 33

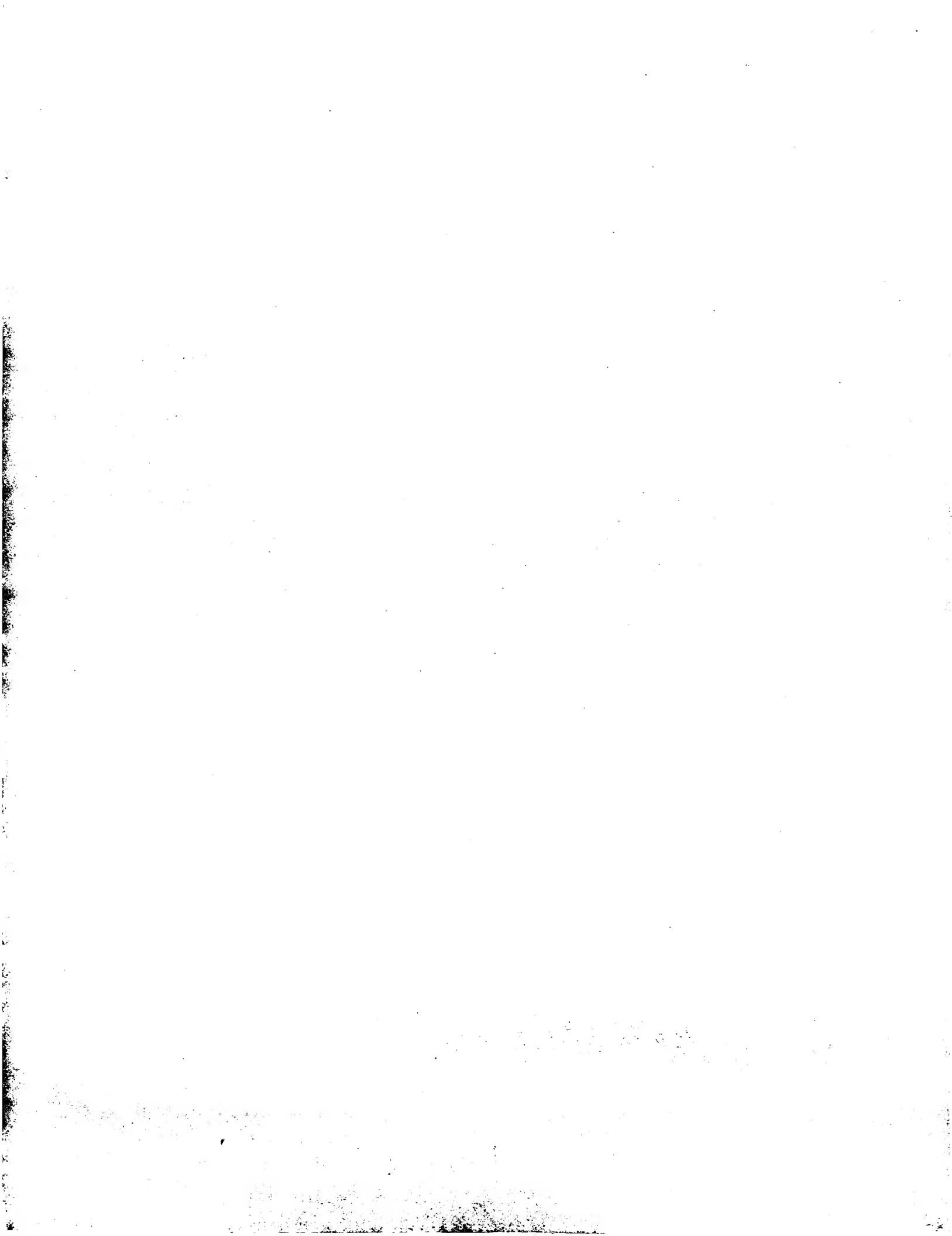


34/38

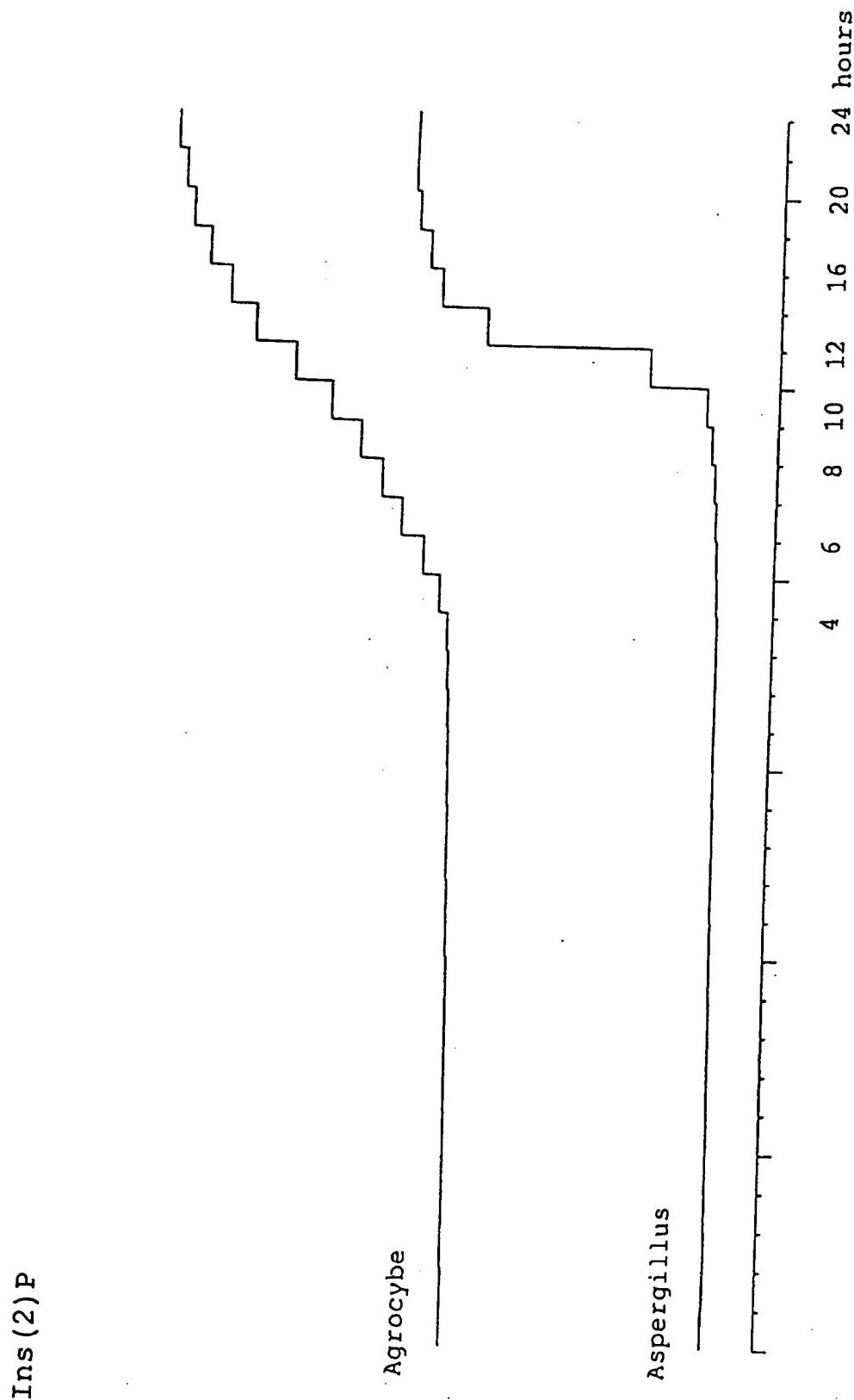


Ins(1,2) P2

Fig. 34

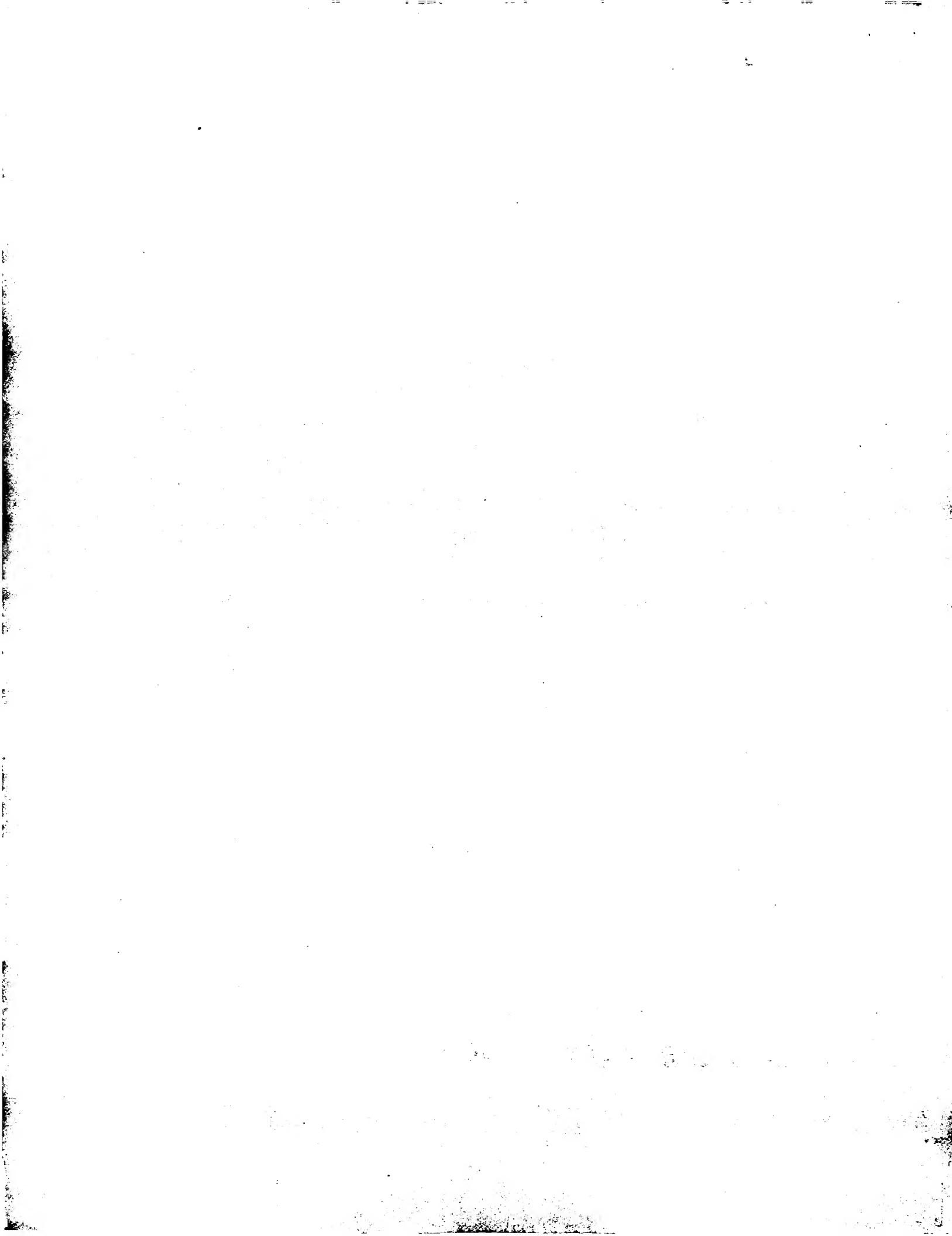


35/38



Ins (2) P

Fig. 35



36/38

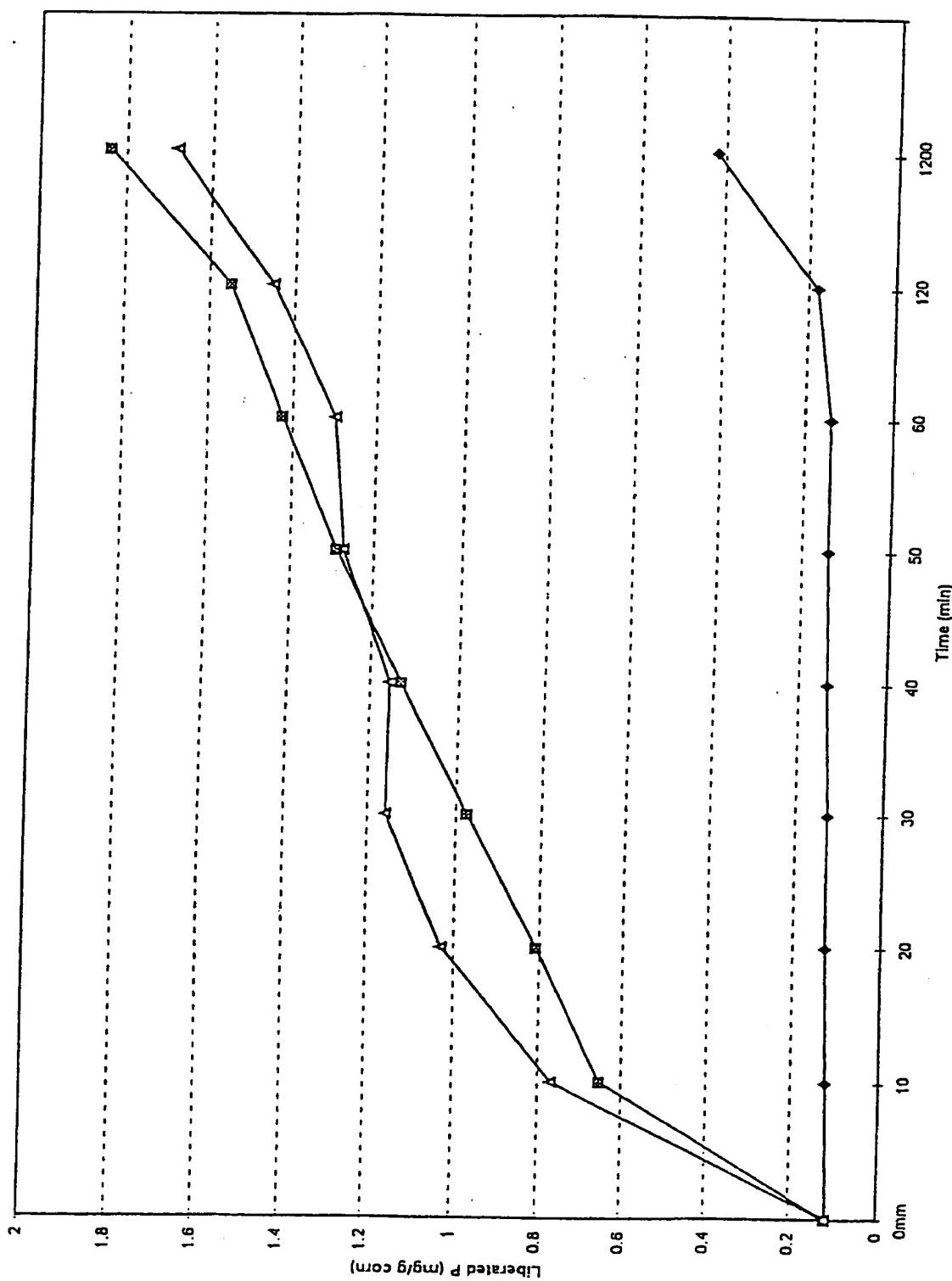
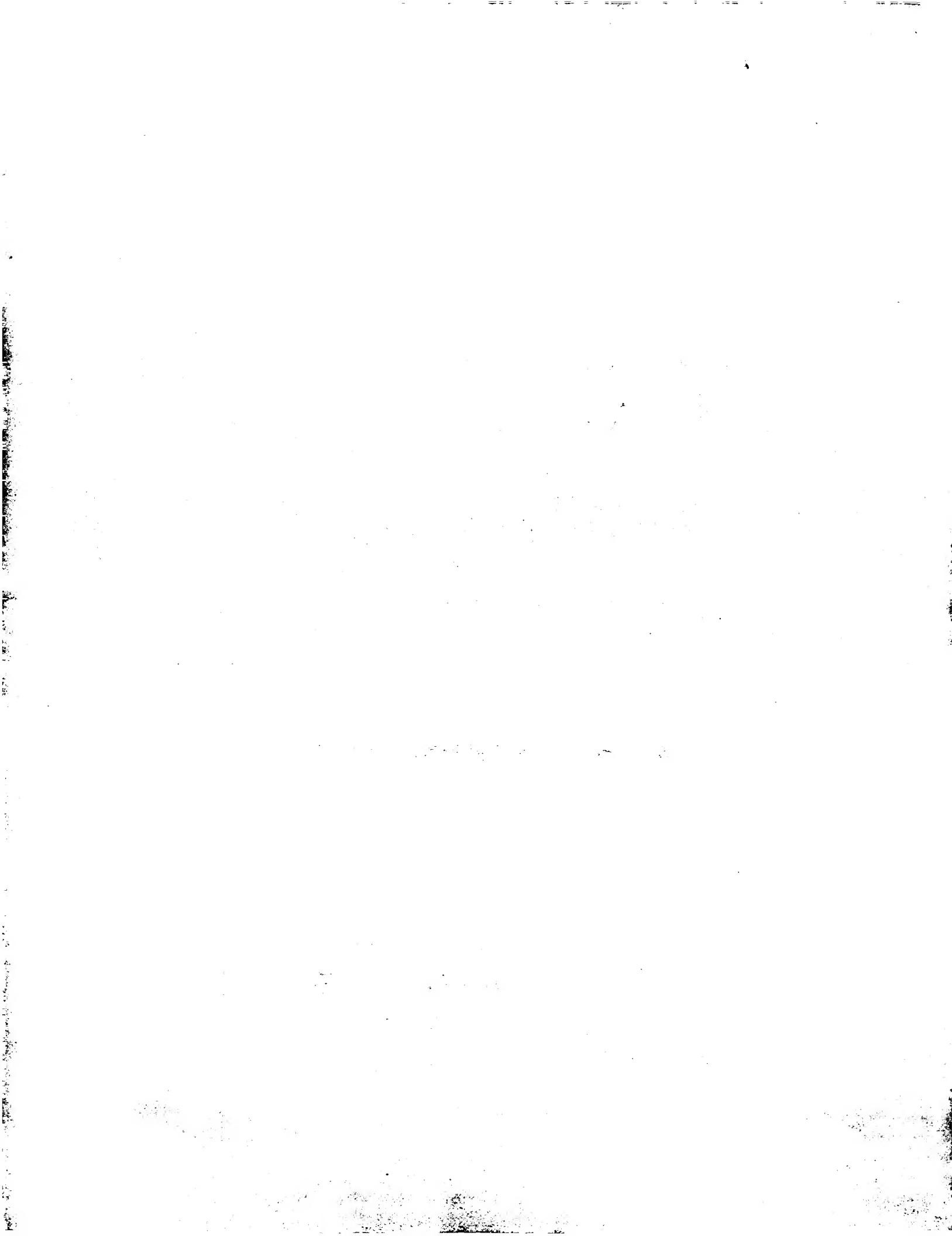


Fig. 36



37/38

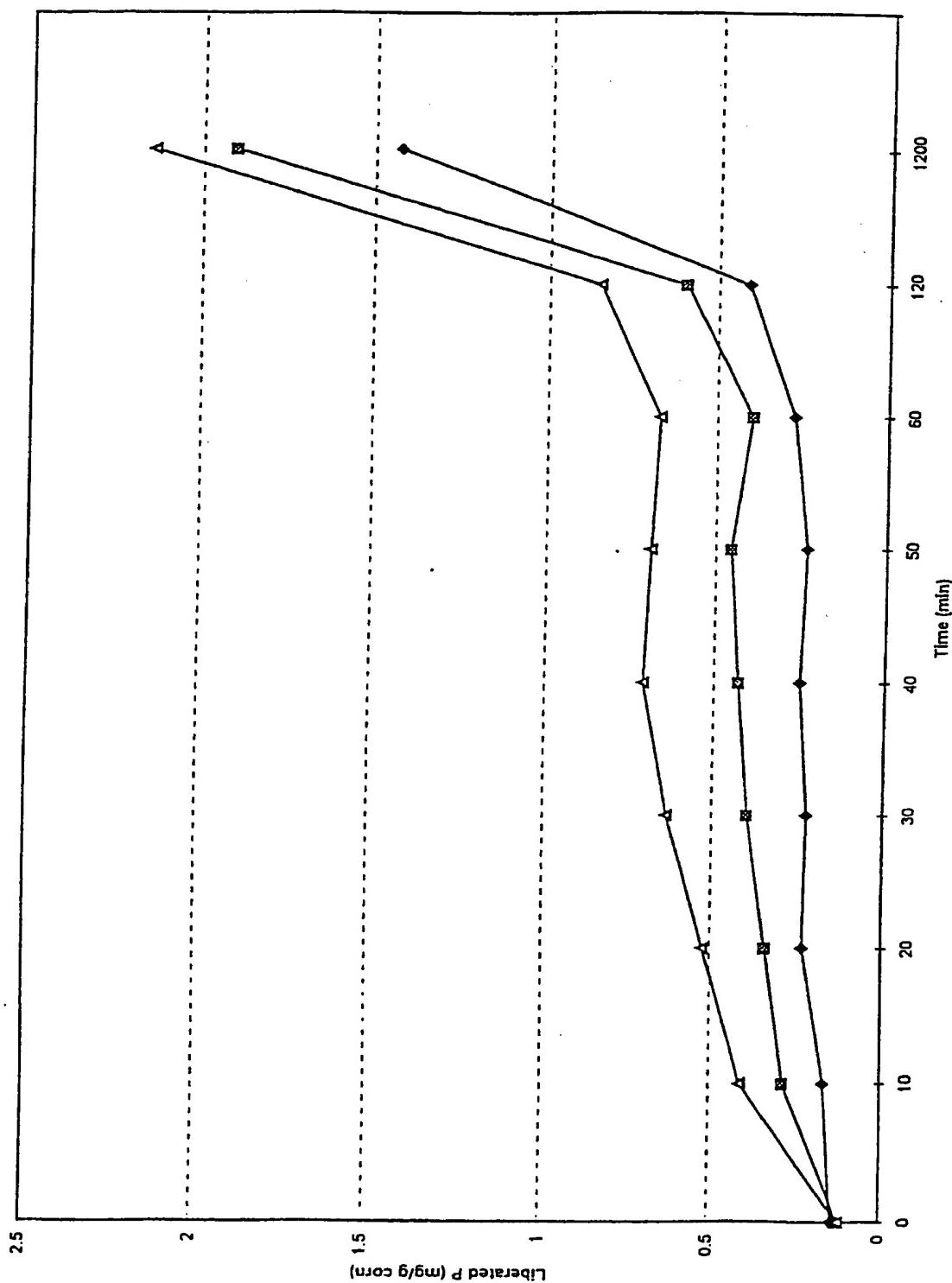
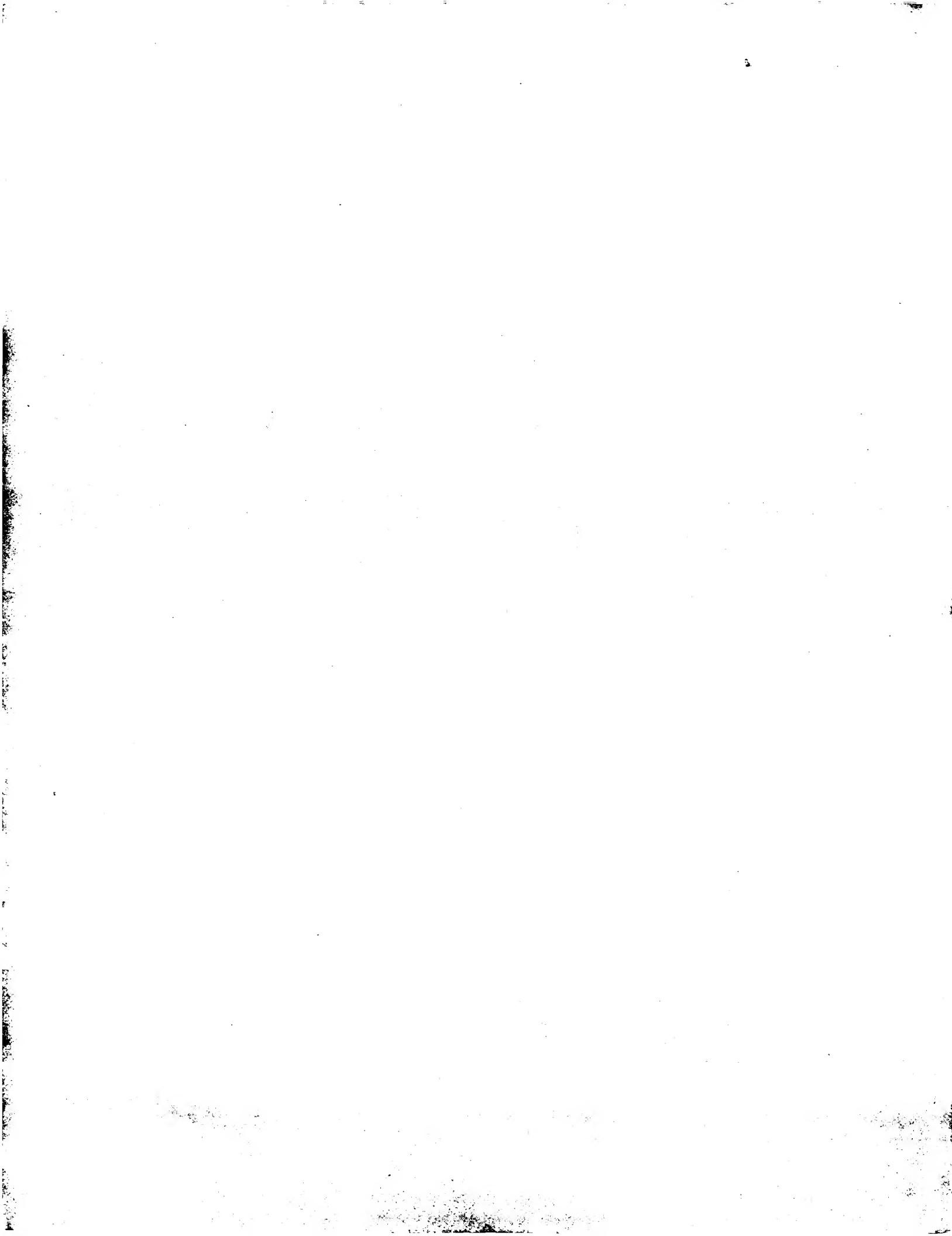
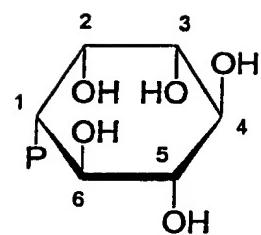


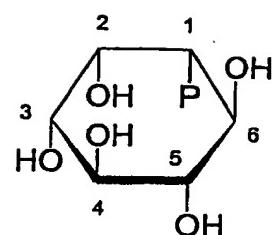
Fig. 37



38/38

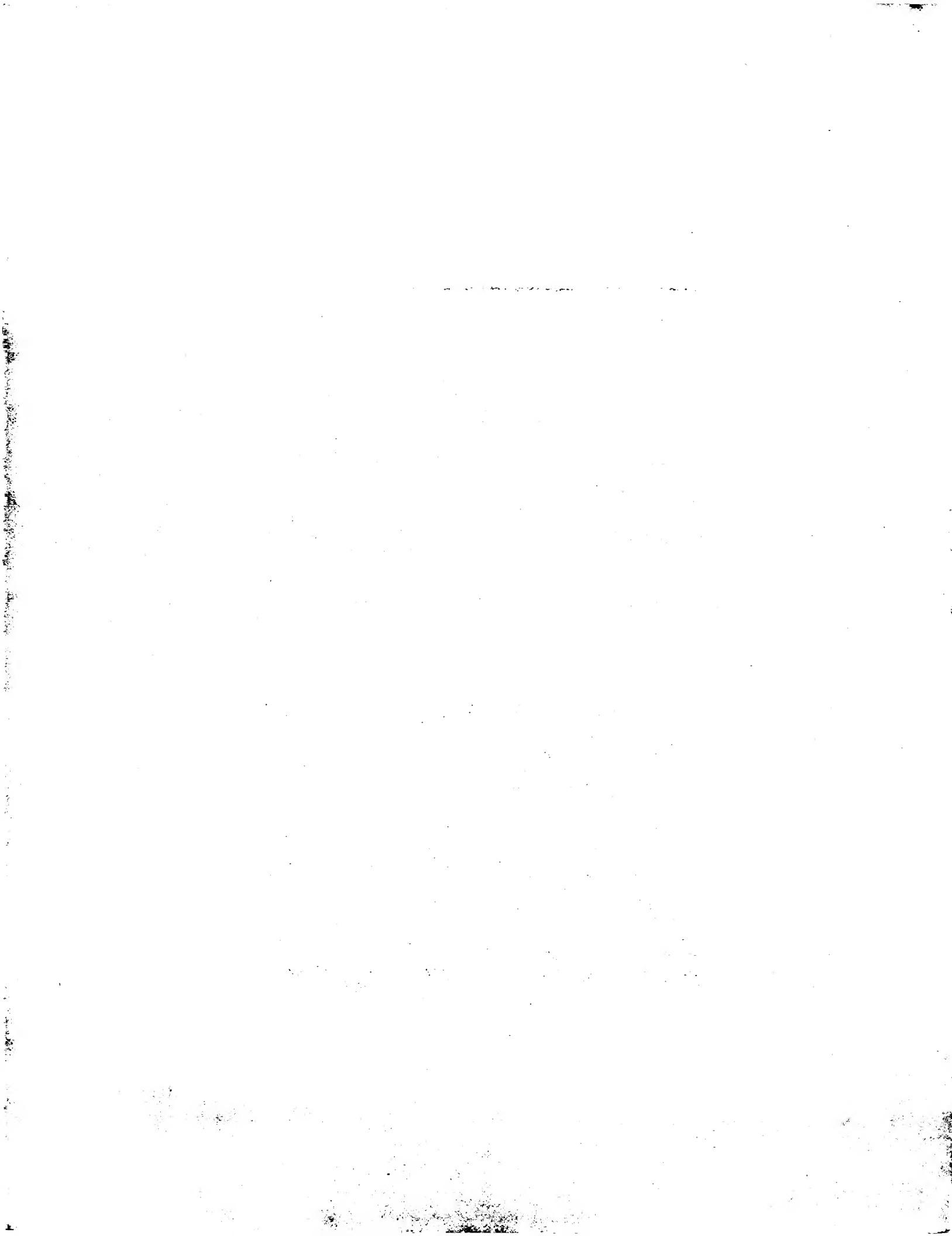


1D-Ins(1)P



1L-Ins(1)P

Fig. 38



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00568

## A. CLASSIFICATION OF SUBJECT MATTER

**IPC6: C12N 9/16 // (C12N 9/16/C12R 1:645)**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

**IPC6: C12N**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

**SE,DK,FI,NO classes as above**

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**WPI, CA, BIOSIS, PIR/SWISSPROT/GENESEQ**

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Die Nahrung, Volume 39, No 5/6, 1995, G. B. Quaglia et al, "Biomass and hydrolytic and oxidative enzymes production by fungal growth on wheat milling by-products" page 483 - page 489 --	1-43
X	EP 420358 A1 (GIST-BROCADES N.V), 3 April 1991 (03.04.91), see sequence; & Geneseq database, No R11333, 31 May 1991 --	2-3
A	Dialog Information Services, file 5, BIOSIS, Dialog accession no. 10480269, Biosis no. 96080269, McElhinney C et al: "Phosphatase activity of four ectomycorrhizal fungi found in a sitka spruce- -japanese larch plantation in ireland"; & Mycol Res 97 (6). 1993. 725-732 --	1-43

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

30 March 1998

02-04-1998

Name and mailing address of the ISA/  
Swedish Patent Office  
Box 5055, S-102 42 STOCKHOLM  
Facsimile No. +46 8 666 02 86

Authorized officer

Yvonne Siösteen  
Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00568

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Enzyme Microb. Technol., Volume 5, Sept 1983, S. J. Howson et al, "Production of phytate-hydrolysing enzyme by some fungi" page 377 - page 382	1-43
A	--  PIR Database, Pirl:Jn0482, Accession no. JN0482; PN0023, Ullah, A.H.J. et al: "Aspergillus ficuum phytase; Complete primarystructure elucidation by chemical sequencing", 30 Sep 1993; & Biochem. Biophys. Res. Commun. 192, 747-753, 1993	1-43
A	--  EP 0684313 A2 (F. HOFFMANN-LA ROCHE AG), 29 November 1995 (29.11.95), & Geneseq, R88623, 26 March 1996	1-43

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00568

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00568

The invention claimed relates to isolated phytases being derived from Basidiomycota. The phytases are characterized by having an increased heat stability and faster release of phosphate from phytate.

Unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features"- i.e. features that define a contribution which each of the inventions makes over the prior art. (See Annex B to Administrative Instructions and Rule 13.2).

Quaglia, in Die Nahrung, 39, 1995, p 483-489 discloses phytase from Basidiomycetes. Therefore, phytases from Basidiomycetes cannot be seen as a unifying novel technical feature.

Accordingly, each of the 14 sequences of claim 1 is considered as one invention.  
It has not been made probable that each of the small sequences of claim 1 would lead to a phytase having a common technical feature.

Although the claims are composed of different inventions they have all been searched without inviting the applicant to restrict the claims or to pay additional fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

02/03/98

International application No.

PCT/DK 97/00568

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 420358 A1	03/04/91	NONE	
EP 0684313 A2	29/11/95	CN 1126243 A JP 8056676 A	10/07/96 05/03/96

